Universidade de Aveiro Departamento de Química
Ano 2017

Diogo Cerqueira Queirós

Valorização de resíduos industriais através da eco-engenharia de culturas mistas

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Engenharia Química, realizada sob a orientação científica da Doutora Luísa S. Serafim, Professora Auxiliar do Departamento de Química da Universidade de Aveiro, e da Doutora Simona Rossetti, Investigadora do Istituto di Ricerca sulle Acque, Roma.

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"Thank you, Mr. Secretary General, for the honor to address this body once more. And thanks to the distinguished climate leaders assembled here today who are ready to take action.

President Abraham Lincoln was also thinking of bold action 150 years ago when he said:

"The dogmas of the quiet past are inadequate to the stormy present. As our case is new so we must think anew and act anew. We must disenthrall ourselves and then we shall save our country."

He was speaking before the US Congress to confront the defining issue of his time – slavery.

Everyone knew it had to end but no one had the political will to stop it. Remarkably, his words ring as true today when applied to the defining crisis of our time – Climate Change.

As a UN Messenger of Peace, I have been travelling all over the world for the last two years documenting how this crisis is changing the natural balance of our planet. I have seen cities like Beijing choked by industrial pollution. Ancient Boreal forests in Canada that have been clear cut and rainforests in Indonesia that have been incinerated. In India I met farmers whose crops have literally been washed away by historic flooding. In America I have witnessed unprecedented droughts in California and sea level rise flooding the streets of Miami. In Greenland and in the Arctic I was astonished to see that ancient glaciers are rapidly disappearing well ahead of scientific predictions. All that I have seen and learned on this journey has terrified me.

There is no doubt in the world's scientific community that this a direct result of human activity and that the effects of climate change will become astronomically worse in the future.

I do not need to throw statistics at you. You know them better than I do, and more importantly, you know what will happen if this scourge is left unchecked. You know that climate change is happening faster than even the most pessimistic of scientists warned us decades ago. It has become a runaway freight train bringing with it an impending disaster for all living things.

Now think about the shame that each of us will carry when our children and grandchildren look back and realize that we had the means of stopping this devastation, but simply lacked the political will to do so.

Yes, we have achieved the Paris Agreement. More countries have come together to sign this agreement today than for any other cause in the history of humankind – and that is a reason for hope – but unfortunately the evidence shows us that it will not be enough.

Our planet cannot be saved unless we leave fossil fuels in the ground where they belong. An upheaval and massive change is required, now. One that leads to a new collective consciousness. A new collective evolution of the human race, inspired and enabled by a sense of urgency from all of you.

We all know that reversing the course of climate change will not be easy, but the tools are in our hands – if we apply them before it is too late.

Renewable energy, clean fuels, and putting a price on carbon pollution are beginning to turn the tide. This transition is not only the right thing for our world, but it also makes clear economic sense, and is possible within our lifetime.

But it is now upon you to do what great leaders have always done: to lead, inspire, and empower as President Lincoln did in his time.

We can congratulate each other today, but it will mean nothing if you return to your countries and fail to push beyond the promises of this historic agreement. Now is the time for bold unprecedented action.

My friends, look at the delegates around you. It is time to ask each other – which side of history will you be on?

As a citizen of our planet who has witnessed so much on this journey I thank you for all you have done to lay the foundation of a solution to this crisis, but after 21 years of debates and conferences it is time to declare no more talk. No more excuses. No more ten-year studies. No more allowing the fossil fuel companies to manipulate and dictate the science and policies that effect our future. This is the only body that can do what is needed. You, sitting in this very hall.

The world is now watching. You will either be lauded by future generations, or vilified by them.

Lincoln's words still resonate to all of us here today:

"We will be remembered in spite of ourselves. The fiery trial through which we pass will light us down, in honor or dishonor, to the last generation… We shall nobly save, or meanly lose, the last best hope of earth."

That is our charge now – you are the last best hope of Earth. We ask you to protect it. Or we – and all living things we cherish – are history.

Thank you."

Leonardo DiCaprio
UN Messenger of Peace with a special focus on climate change, April 12th 2016

jury

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However, there are a few people that I must mention. To Alexandre Fonseca, Catarina Rangel, Eduarda Morais, Rita Sousa and Joana Pereira, thank you for trusting in me to help you guide through your master's project and for your contribution for this thesis. Without you, all of this wouldn't be possible. Thank you for growing with me.

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To my parents, o "Sr. Queirós e a D. Irene"; to my sister, Mariana; and to my girlfriend, Sara: Thank you for being there, no matter what!

palavras-chave

Polihidroxialcanoatos, Ácidos Orgânicos de Cadeia Curta, Culturas Microbianas Mistas, Resíduos Industriais, Licor de Cozimento ao Sulfito Ácido, Valoriação, Sustentabilidade, Biorrefinaria

resumo

Hoje em dia, há uma necessidade urgente de reinventar os processos industriais a partir dos quais a nossa sociedade subsiste, tornando-os mais sustentáveis. A União Europeia (UE) estabeleceu um conjunto de pacotes com medidas que abrangem todo o ciclo: desde a produção ao consumo e à gestão de resíduos, e quanto ao mercado de matérias-primas secundárias. Pretende-se, com isto, caminhar em direção a uma economia circular, dando um destino diferente a todos os resíduos gerados.

Em Portugal, umas das maiores e principais atividades económicas diz respeito à indústria papeleira. Consequentemente, grandes quantidades de resíduos são geradas. Estes são aproveitados para a produção de energia ou químicos para betumes e colas. Contudo, outras vias de valorização devem ser exploradas.

São dois os principais métodos usados para a produção de papel: Kraft ou Cozimento ao Sulfito Ácido. O último dá origem ao licor de cozimento ao sulfito ácido (LCSA), rico em lenhosulfonatos (LS), compostos fenólicos e derivados, açúcares, como xilose e glucose, e ácido acético.

Apesar da fração significativa de compostos recalcitrantes e inibidores, o LCSA pode ser valorizado através de processos microbiológicos. Biopolímeros, como polihidroxialcanoatos (PHA), substratos microbianos, e *building-blocks*, como ácidos orgânicos de cadeia curta (AOCC), podem ser produzidos.

Os PHA são um grupo promissor de polímeros degradáveis com potencial para substituir alguns dos termoplásticos mais comuns. Nos últimos anos, a investigação tem-se focado no desenvolvimento de alternativas à produção deste polímero, incluindo o uso culturas microbianas mistas (CCM) e de resíduos industriais como substrato. O uso de CCM faz com que não sejam precisas condições de esterilidade e permite o uso de equipamento mais barato. O ponto fulcral para a produção de PHA a partir de CCM é a seleção da cultura. Normalmente, esta é feita submetendo a cultura a períodos de alternância de disponibilidade de carbono, designada alimentação aeróbia dinâmica (AAD).

Os AOCC são monoácidos alifáticos com uma vasta gama de aplicações na indústria alimentar, farmacêutica e química. Desempenham, também, um importante papel enquanto compostos intermediários em processos biológicos, como a produção de PHA.

Neste trabalho, o processo de produção de PHA a dois e três passos, usando o LCSA, foi estudado. O processo a dois passos incluiu (1) o enriquecimento da CCM em microrganismos (MO) acumuladores de PHA, usando diretamente o LCSA, seguido de (2) maximização da produção, usando a cultura selecionada e o LCSA. No processo a três passos, um passo preliminar de acidificação foi introduzido antes da seleção, com vista à conver-

Resumo (continuação)

são dos açúcares do LCSA em AOCC. Todos os passos, e respetivos impactos nos passos seguintes, foram estudados. Paralelamente, a caracterização da comunidade microbiana do passo de seleção foi realizada, de forma a afinar os parâmetros operacionais para melhorar a produção de PHA.

O processo a dois passos levou à seleção de uma cultura com baixa capacidade acumulativa, apesar da estabilidade do reator. Para além disso, os longos períodos necessários à estabilização (250 dias) constituem um obstáculo à viabilidade do processo. Posteriormente, uma extensa caracterização microbiológica revelou uma população paralela incapaz de acumular PHA. Esta população pode ter subsistido graças à fração de açúcares, a qual uma CCM não consegue converter em PHA.

Para reduzir o tempo de estabilização do reator de seleção e entender o efeito dos AOCC na seleção de MO acumuladores de PHA, o LCSA foi suplementado com ácidos sintéticos. Obteve-se, assim, uma cultura estável com apenas 25 dias de operação. Adicionalmente, a cultura selecionada conseguiu acumular um máximo de 47.1%, 7 vezes mais elevado do que aquele obtido nas condições anteriores. A comunidade microbiológica era dominada por *Betaproteobacteria*, nomeadamente, *Acidovorax* (71%).

Uma vez provada a relação entre estabilidade do reator e a presença de ácidos, o passo seguinte focou-se na fermentação acidogénica do LCSA. Ao invés do uso de uma típica cultura anaeróbia, foi possível selecionar MO acidogenos, capazes de converter os açúcares em AOCC, a partir de uma cultura aeróbia. O processo decorreu num reator contínuo de mistura perfeita (RCMP) sem controlo de pH. Obteve-se, desta forma, um máximo de 7.45 gCOD L^{-1} de ácidos, correspondendo a um grau de acidificação de 38.0%.

Na produção de AOCC, a influência do pH e da conformação do reator foi analisada. Controlando o pH a 6, 7 e 8 num RCMP obteve-se perfis variados de ácidos com uma concentração de 2.36, 2.38 e 2.27 gCOD L^{-1}, respetivamente. Alterando a conformação do reator para reator contínuo de leito fixo, obteve-se um perfil de ácidos dominado pelo ácido butírico. Caracterizou-se, igualmente, por uma maior estabilidade do que o RCMP sem controlo de pH.

Por fim, o processo a três passos foi estudado. O efluente recolhido dos reatores de acidogénese foi usado para selecionar uma CCM. Uma vez mais, não demorou mais que 25 dias a alcançar uma fase estável de cada vez que os parâmetros operacionais eram manipulados (tempo de retenção hidráulico, carga orgânica e/ou duração do ciclo). Uma cultura robusta foi obtida com uma boa capacidade acumulativa de PHA usando diferentes resíduos. Um máximo de 74.4% de PHA foi alcançado sob limitação por azoto. Diferentes copolímeros e homopolímero foram produzidos através do uso de diferentes resíduos.

keywords

Polyhydroxyalkanoates, Short-Chain Organic Acids, Mixed Microbial Cultures, Industrial byproducts, Hardwood Sulfite Spent Liquor, Valorization, Sustainability, Biorrefineries

abstract

Nowadays, there is an urgent need to reinvent the industrial processes in which our society relies, making them more sustainable. The European Union (EU) established a set of packages with measures covering the whole cycle: from production and consumption to waste management and the market for secondary raw materials. It is intended to move from a “cradle-to-grave” tendency, and start to give a different destination to all residues and wastes generated.

In Portugal, one of the main and biggest activities is the pulp and paper industry. Consequently, large quantities of residues are generated and are usually burnt for energy or chemicals recovery and for concretes additives and adhesives, but other ways to valorize them are needed.

Two main process are used for pulp making: kraft and acidic aulfite pulping. The latter originates hardwood sulfite spent liquor (HSSL) which is characterized for having significant amounts of lignosulphonates (LS), phenolic extractives compounds and derivatives, sugars such as xylose and glucose and acetic acid.

Despite its considerable fraction of recalcitrant and inhibitory components, HSSL can be valorized through microbial conversion. Biopolymers such polyhydroxyalkanoates (PHA) and microbial substrates and building blocks like short-chain organic acids (SCOA) can be produced.

PHA are a promising group of eco-efficient biodegradable polymers with potential to replace some of the most commonly used thermoplastics. In recent years, research focused on the development of alternative PHA production processes, including the use waste/surplus based feedstocks and mixed microbial cultures (MMC). MMC do not require sterilization and expensive equipment, thereby reducing costs. The key point of PHA production by MMC is culture selection, which is generally carried out by subjecting cultures to alternate periods of carbon availability, designated as aerobic dynamic feeding (ADF).

SCOA are aliphatic monocarboxylic acids, with a vast number of applications in food and beverages industry, pharmaceutical and chemical fabrication fields. Also, they have an important role as intermediates in many biological processes, such PHA production.

In this thesis, two and three-stage PHA production processes from HSSL were studied. The two-stage process included (1) the enrichment of an MMC in PHA-storing microorganisms (MO) direct from HSSL, followed by (2) maximization of PHA production using the selected culture and the HSSL. In the three-stage, a preliminary step of acidification, where the sugars fraction is converted into SCOA was introduced before the selection step. All stages were studied and the respective impact of each stage on subsequent ones was also

abstract (continuation)

assessed. In addition, microbial community characterization of the selection stage was done to tune operational parameters towards the improvement of PHA accumulation.

The two-step process led to a culture with low PHA storage performance, despite the stability of the selection reactor. Furthermore, the long stabilization time of the process (250 days) required could be a significant obstacle to the viability of the process. Later, an extensive microbial characterization revealed a side population that might be responsible to hinder the selection step stopping the dominance of PHA-storing MO. Such fact, could be due to the sugars fraction that MMC are not able to convert into PHA.

To decrease the stabilization time and understand the effect of SCOA in the PHA-storing MO selection, HSSL was supplemented with synthetic SCOA. A stable MMC was reached after only 25 days. Additionally, the selected MMC reached a maximum PHA content of 47.1%, which was 7 times higher than in the previous condition. The selection step led a community dominated by *Betaproteobacteria*, namely *Acidovorax* (71%).

Once proved that the single addition of SCOA led to stable cultures, the next step focused on the acidogenic fermentation of HSSL. Instead of using a typical anaerobic culture, from an aerobic MMC inoculum was possible to successfully select acidogenic microorganisms able convert sugars into SCOA without pH control in a continuous stirring tank reactor (CSTR). The maximum concentration of SCOA produced was 7.45 gCOD L^{-1}, corresponding to a degree of acidification 38.0%.

In SCOA production, pH and reactor conformation influence was also assessed. Controlling pH at 6, 7 and 8 in the CSTR resulted in diverse SCOA profiles with average productions of 2.36, 2.38 and 2.27 gCOD L^{-1}, respectively. Changing the reactor conformation from CSTR to moving bed biofilm reactor (MBBR) led to a profile dominated by butyric instead of acetic acid, with a more stable production than the CSTR without pH control.

Finally, the three-stage process was applied. The effluent collected from the acidogenic fermentation was used to enrich an MMC. It took no longer than 25 days to reach a stable phase every time an operational parameter (HRT, OLR and/or cycle length) was changed. A robust culture was obtained, that despite being selected with only one of the previous effluents, it could accumulate PHA when different streams from the pulp industry were fed. A maximum of PHA accumulation of 74.4% was reached, under ammonium limitation. Moreover, different copolymers and homopolymer were obtained through the feeding of the different streams.

Contents

Figures Index

Tables Index

Abbreviations

3H2MB	3-hydroxy-2-metilbutyrate
3H2MV	3-hydroxy-2-metilvalerate
3HAME	3-hydroxyalkanoate methyl ester
3HB	3-hydroxybutyrate
3HDD	3-hydroxydodecanoate
3HHp	3-hydroxyheptanoate
3HHx	3-hydroxyhexanoate
3HMBE	3-hydroxybutyrate methyl ester
3HN	3-hydroxynonanoate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
Acet	Acetic Acid
ADF	Aerobic Dynamic Feeding
AD	Acidification Degree
AF	Acidogenic Fermentation
AN/AE	Anaerobic/Aerobic Process
AN/AO	Anaerobic/Anoxic Process
AnD	Anaerobic Digestion
AS-SBR	Activated Sludge Sequenced Batch Reactor
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
BTX	Benzene, Toluene, and Xylene
CDW	Cell Dry Weight
C/N	Carbon-to-Nitrogen Ratio
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred Tank Reactor
DGGE	Denaturing Gradient Gel Electrophoresis
DO	Dissolved Oxygen Concentration
EBPR	Enhanced Biological Phosphorus Removal

EOD	Earth Overshoot Day
FF	Feast and Famine
F/F	Feast-to-Famine ratio
FISH	Fluorescence *in situ* Hybridization
GAO	Glycogen Accumulating Organism
GB	Glucose Biopolymer
HRT	Hydraulic Retention Time
HSSL	Hardwood Spent Sulfite Liquor
IFAS	Integrated biofilm activated sludge
LCL	long-chain-length
LS	Lignosulphonates
MAAS	Microaerophilic– aerobic system
MBBR	Moving Bed Biofilm Reactor
MCL	medium-chain-length
MMC	Mixed Microbial Cultures
MRM	Microbial Resource Management
NADH	Nicotinamide Adenine Dinucleotide
NREL	American National Renewable Energy Laboratory
OLR	Organic Loading Rate
OME	Olive Mill Efluent
OTU	Operational Taxonomic Units
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-Hydroxyvalerate)
PCA	Principle Components Analysis
PCR	Polymerase Chain Reaction
PHB	Polyhydroxybutyrate
PAO	Polyphosphate Accumulating Organism
PHA	Polyhydroxyalkanoate
PHA_{SCL}	short-chain-length polyhydroxyalkanoate
PHA_{MCL}	medium-chain-length polyhydroxyalkanoate

PHA_{LCL}	long-chain-length polyhydroxyalkanoate
PLA	Poly(lactic acid)
PS	Primary Sludge
PSS	Pseudo steady-state
qPCR	Quantitative Polymerase Chain Reaction
RDP	Ribosomal Database Project
RISA	Ribosomal Intergenic Spacer Analysis
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RuMP	Ribulose MonoPhosphate
SBR	Sequenced Batch Reactor
SCOA	Short-chain Organic Acid
SCL	Short-chain-length
SRT	Sludge Retention Time
S	Substrate
SS	Suspended solids
SSL	Sulfite Spent Liquor
TCA	Tricarboxylic Acid
TFO	Tetrad Forming Organisms
T-RF	Terminal-Restriction Fragment
T-RFLP	Terminal-Restriction Fragment Length Polymorphism
TSS	Total Suspended Solids
VSS	Volatile Suspended Solids
WAS	Waste Activated Sludge
WWTP	Wastewater Treatment Plants
Xyl	Xylose

Kinetic and Stoichiometric Parameters

$-q_{Xyl}$	Xylose specific consumption rate
$-q_{Acet}$	Acetic acid specific consumption rate
$-q_{Prop}$	Propionic acid specific consumption rate
$-q_{But}$	Butyric acid specific consumption rate
$-q_{Val}$	Valeric acid specific consumption rate
r_{Acet}	Acetic acid volumetric consumption rate
r_{Prop}	Propionic acid volumetric consumption rate
r_{But}	Butyric acid volumetric consumption rate
r_{Val}	Valeric acid volumetric consumption rate
$-q_S$	Substrate specific consumption rate
$-q_{SCOA}$	Short-chain organic acids specific consumption rate
q_{pHA}	HA specific production rate
q_{PHA}	PHA specific production rate
q_{pGB}	GB specific production rate
q_{VolPHA}	PHA volumetric production rate
q_{HB}	HB volumetric production rate
q_{HV}	HV volumetric production rate
OUR	Oxygen uptake rate
$Prod_{Esp}$	Specific productivity
$Prov_{Vol}$	Volumetric Productivity
$Y_{HA/S}$	HA production yield on substrate
$Y_{HA/Acet}$	HA production yield on acetic acid
$Y_{GB/S}$	GB production yield on substrate
$Y_{GB/Acet}$	GB production yield on acetic acid
$Y_{X/S}$	biomass production yield on substrate
μ_{max}	Maximum growth rate

Chapter 1

Introduction and Thesis Outline

1.1. Introduction

August 8th, 2016 marked the day on which humanity's resources consumption exceeded Earth's capacity to regenerate those resources during the year. This day is designated Earth Overshoot Day (EOD) and in 2013, when this work started, it was August 20th, 12 days later than the current year.

Since 1987, more ecological resources and services are used worldwide than nature can regenerate through overfishing, overharvesting forests, and emitting more carbon dioxide into the atmosphere than forests can sequester. In today's world, where humanity is already exceeding planetary limits, ecological assets are becoming more critical. Global Footprint Network updates, each year, the ecological risk profile of each country. Many are running ecological deficits, with footprints larger than their own biological capacity. Others depend heavily on resources from elsewhere, which are under increasing pressure. Portugal possesses a 150% footprint greater than its biocapacity, Fig. 1.1.

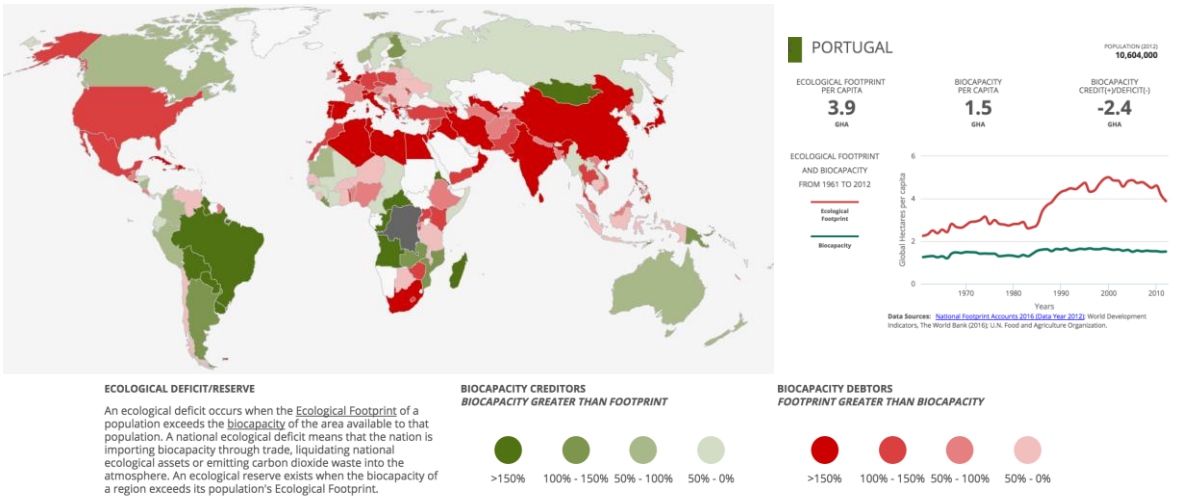

Fig. 1.1. Map representing the biocapacity creditors and debtors, from Global Footprint Network.

To invert the balance, Portugal should reduce its resources dependence and increase and maintain its ecological reserves. To achieve this, a better residue management and valorization to produce and obtain products of interest along the treatment of such wastes is required.

A new industrial trend relies on moving from petroleum-based to biomass-based products and sustainable manufacturing processes. The development of a bio-based product industry offers an economical and environmental friendly solution for the surplus agricultural commodities production with low economic income for farmers and for the large amounts of industrial wastes with high disposal. In this novel bio-based product industry, arose the concept of biorefinery, which was defined by the American National Renewable Energy Laboratory (NREL) as "a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass".

When compared to petroleum refineries, biorefineries can process a wider range of feedstocks using different processing technologies. The productivity and viability of a biorefinery can be enhanced by integrating the production of higher valuable bioproducts into the fuel and power plants. It is also possible to enhance productivity and efficiency resorting to operations that decrease the overall energy intensity of biorefinery's unit operations, once biorefineries can integrate and maximize the use of all feedstock components, by-products and waste streams.

One possible production line, which results from the processes integration, is the production of biomaterials, namely bioplastics, using industrial wastes or by-products (real substrates) as substrate. The use of conventional plastics raised several problems such as disposable concerns, emissions of greenhouse gases, exhaustion and an increase demand for the fossil resources. In recent years, bioplastics, more particular polyhydroxyalkanoates (PHA), produced by mixed microbial cultures (MMC), arose as an alternative. Also, a greener production of short-chain organic acids (SCOA) can be explored. Despite being the preferable precursors for PHA production by MMC, SCOA are mostly used as building blocks by the chemical industry.

Pulp and paper industry represents the 3rd largest industry in Portugal and is one of the dominants in Europe. Despite the Portuguese economic crisis, investments still being done since 2009. The Navigator Company invested 159 million euros to dominate the European market in tissue paper production and ALTRI group invested in the production of soluble paste - used in the textile industry for the manufacture of viscose,

with increasing demands from China. This represents a huge side production of wastes and residues that can be valorizes within the lignocellulosic biorefinary concept.

In this way, this project was idealized and created aiming to investigate the production of PHA and SCOA by an MMC resorting to a by-product from the pulp industry, provided by Caima S.A., Hardwood Spent Sulfite Liquor (HSSL).

The next chapters will provide a suitable contextualization on the PHA and SCOA production as well as the importance of following the bacterial community evolution along the bioreactors operation. The experimental work focused on the selection of PHA-storing MMC and on the several parameters that can be manipulated to achieved such goal. Also, molecular tools were applied to understand the impact of the operational conditions on the selected cultures. Regarding SCOA, the main objective was to prove the possibility to obtain them from HSSL via acidogenic fermentation and which SCOA would be obtained if pH and reactor configuration would be manipulated.

1.2. Thesis Outline

This thesis is composed of 11 chapters, including the current introductory chapter describing the motivation and the outline of the work developed during the doctorate project.

Chapter 2 intends to contextualize the use of MMC for PHA production, by describing the different approaches and comparing them with the traditional processes employing pure or genetically modified organism. Moreover, the necessity of PHA development are remarked and their properties, synthesis and applications were described (published as a book chapter, in Recent Advances in Biotechnology). Also, a short state of the art regarding acidogenic fermentation is included.

Chapter 3 aims the isolation and characterization of organisms able to store PHA, from an MMC selected under feast and famine conditions, using HSSL as carbon source. The search for robust strains able to resist and metabolize inhibitory compounds could be a path to conjugate renewable carbon sources with high productivities typical of pure cultures (published at Annals of Microbiology).

Chapter 4 infers the possibility to establish a steady process with a robust microbial community able to deal with the fluctuations of the HSSL composition, within a 2-step process (published at Journal of Chemical Technology and Biotechnology).

Chapter 5 presents and extensive molecular characterization of the culture selected in the previous chapter using fluorescence *in situ* hybridization, denaturing gradient gel electrophoresis and 16S rDNA clone library.

Chapter 6 explores the behavior of an MMC fed with a SCOA-enriched HSSL. Given the low accumulation obtained in chapter 4 due to a high dynamic culture with a considerable non-storing PHA side population (unveiled at chapter 5), a different strategy was adopted. SCOA-enriched HSSL was used to lower the acclimatization time and improve the performances of PHA production.

In *Chapter 7*, different adsorbents were used to decrease the HSSL toxicity and ease further bioprocesses. Lignosulphonates, phenolic compounds and its derivatives were the main cause of the instability observed in the previous chapters. An attempt to develop a new method to be used as pre-treatment was done.

HSSL was used as substrate for the production SCOA through acidogenic fermentation, in *Chapter 8*. These chemical compounds bear an important and wide range of applications in the production of biopolymers, bioenergy and in biological removal of nutrients from wastewaters

Following the results obtained in the previous chapter, *Chapter 9* deepens the influence of operational parameters on the SCOA concentration and distribution. Parameters as pH and reactor conformation were studied in detail.

Finally, *Chapter 10* studies the use of different pulp and paper waste streams for PHA-storing MMC enrichment and to manipulate its PHA production. Such streams were submitted to acidogenic fermentation of HSSL in chapter 9, and used to manipulate the PHA composition.

Chapter 11 is a general conclusion and a future perspective summary.

Chapter 2

PHA and SCOA production: A way to valorize industrial wastes

PHA production by MMC became the central point of many research works. It became a process to be considered for polymer production intended to replace petrochemical derived plastics. The ability to store PHA provides microbial communities a competitive advantage for survival under transient conditions typical of waste treatment systems. In this way, MMC can continuously adapt to the operational conditions increasing the number PHA-storing organisms with minimum requirements of sterility. MMC can produce PHA using waste or industrial by-products as substrates, allowing for their valorization. The use of waste, together with the lower requirements in sterility and process control, could signify a decrease on PHA production costs. The price is one of the main drawbacks that prevent the increase of world PHA market share. This chapter intends to contextualize the use of MMC for PHA production, by describing the different approaches and compare them with the traditional processes employing pure or genetically modified organisms. In parallel, it is explored the feasibility of acidogenic fermentation of such wastes, to produce short-chain organic acids, the preferential MMC substrate.

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2.1. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are a family of polyesters synthesized by several microorganisms showing a rich structural variation. PHA can be composed by different monomeric units known as hydroxyalkanoates (HAs) that can be aliphatic or aromatic (Ward and O'Connor, 2005). Until today, there are more than 150 HAs described, although the 3-hydroxybutyrate (3HB) is the most frequent one. Fig. 2.1 shows the general chemical structure of PHA.

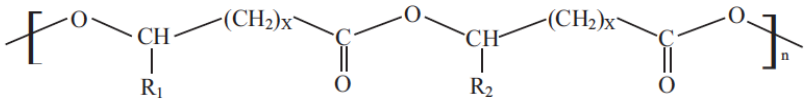

Fig. 2.1. General structure of PHA (R1/R2 = alkyl groups C1–C13, X = 1–4, n = 100–35000 (adapted from Akaraonye et al. (2010)).

The *R* and *X* represented in Fig. 2.1 refer to the length of the side chain (may vary from a methyl to a tridecyl that can contain unsaturations, aromatic groups and halogenated elements) and the size of the alkyl group, respectively. Both *R* and *X* determine the type of HA monomer unit.

When the total number of carbon atoms in the HA monomer unit equals less than five, the HA is known as a short chain-length (SCL) monomer and the PHA resulting from the polymerization of SCL monomers are known as PHA_{SCL}. When the total number of carbon atoms in the HA monomer unit is at least six and up to 14, the HA is known as a medium chain-length (MCL) monomer and the PHA resulting from the polymerization of MCL monomers are known as PHA_{MCL}. And finally, when the monomer has more than 14 carbons, the HA is known as long chain-length (LCL) monomer and the resulting PHA is known as PHA_{LCL} (Akaraonye et al., 2010).

Variations in the length and composition of available side chains lead to innumerous variety of structures. In terms of monomeric composition, PHA can be usually produced as homopolymers, copolymers, and terpolymers. Among copolymers and terpolymers, the organization of monomers can lead to block or random polymers. The structure and composition of the synthesized PHA is known to depend on the type of

microorganism, the raw material used as a carbon source, growth conditions as well as the metabolic pathways. This enables to obtain polymers that can widely vary in terms of physical properties, which allows for a large set of potential applications. Whereas SCL monomers confer to the polymer a high crystallinity, tensile strength, and low elongation at break, MCL monomers, in other hand, confer elasticity, a reduction of crystallinity and melting temperature and improved elongation at break Table 2.1. Laycock et al. (2013) reviewed extensively the properties of PHA biologically produced.

Table 2.1. Comparative explanation on the properties of common plastics and PHA polymers (Gholami et al., 2016; Kumar et al., 2015).

Property	PP	LDPE	PS	PVC	P3HB	P(3HB-*co*-3HV)[a]	P(3HB-*co*-3HHx)	P(3H4HB)
Melting temp. (°C)	168	123	---	---	177	140	127	150
Glass temp. (°C)	-20	-36	90	110	4	-1	-1	-7
Cristalinity (%)	60	30	---	---	70	60	34	45
Young's Modulus (GPa)	1.3	0.4	3.2	3.2	3.5	0.8	0.5	---
Tensile Strength (MPa)	36	20	36	46	43	20	21	104
Elongation break (%)	350	530	2	60	5	50	400	1080
Notched Izod impact strength (J m^{-1})	50	---	24	580	60	110	---	---

PP: Polypropylene; LDPE: Low-density polyethylene; PS: Polystyrene; PVC: Polyvinyl chloride; [a] molar fraction 80:20

All physical properties of PHA described in Table 2.1 closely resemble the properties of synthetic plastics currently dominating the market. Both PHA and synthetic plastics are thermoplastics, moldable, and could be tailor-made for numerous applications ranging from stiff packaging goods to highly elastic materials for coatings (Chen, 2009). In addition, PHA have two qualities that set them apart from conventional plastics: biodegradability and biocompatibility.

Despite their versatility, the major draw-back of PHA is their high production cost, considerably higher than conventional plastics. The final costs of PHA are mainly dependent on the price of substrates added as a carbon source for microbial growth,

estimated to be up to 50% of the final production cost (Możejko-Ciesielska and Kiewisz, 2016). Also, PHA yield on carbon source, PHA productivity and downstream costs (up to 30%) define their position in the global market (Dias et al., 2006; Możejko-Ciesielska and Kiewisz, 2016).

Table 2.2 shows the main prices of industrial PHA produced. Currently, researchers are focusing efforts on reducing production costs making the process economically viable. This is an important effort once the theoretical price of PHA produced is still 3.51 Eur Kg^{-1}, whereas PP or PE cost 1.47 and 1.15 Eur kg^{-1}, respectively (Możejko-Ciesielska and Kiewisz, 2016).

Table 2.2. PHA industrially produced and their current prices.

Polymer	Trade names	Manufactures	Price (kg^{-1})
PHB	Biogreen®	Mitsubishi Gas Chemical Company (Japan)	€ 2.5-3.0
PHB	Mirel™	Telles (US)	€ 1.50
PHB	Biocycle®	PHB Industrial Company (Brazil)	n/a
P(HB-*co*-HV) and PHB	Biomer®	Biomer Inc. (Germany)	€ 3.0-5.0
P(HB-*co*-HV), HBV+ Ecofelx blend	Enmat®	Tianan Biologic Ningbo (China)	€ 3.26
P(HB-*co*-HV)	Nodax™	P&G(US)	€ 2.50
P(HB-*co*-HV)	Nodax™	Lianyi Biotech (China)	€ 3.70
P(HB-*co*-HV)	Kaneka PHBH	Kaneka Corporation	n/a
P(3HB-*co*-4HB)	Green Bio	Tianjin Gree Bio-Science (Japan)	n/a
PHA from P&G	Meredian	Meredian (US)	n/a

The conversion of raw products into biopolymers, along the downstream strategy, seems to be a major contribution to the development of a sustainable biotechnological process and a solution to the cost limitations. Depending on their final applications, the use of MMC present itself as a viable alternative if a treatment-valorization view is adopted, since a huge range of residues, by-products and wastewaters can be used as carbon source, and the equipment and energy needed is significantly lower than those required by processes using pure cultures.

2.1.1. PHA-storing Mixed Microbial Culture

It is common knowledge that PHA-producing genera are quite ubiquitous in Nature. So far, more than 300 were found and part of them cultivated but it is believed that a larger number still remains unknown (Chanprateep, 2010; Dias et al., 2006). Until now, the search for pure and defined cultures with a high PHA-storing capacity has been an objective of most of the researchers working on this field. However, in nature, microorganisms live in community with different relationships among them. Most of the times, the isolation of bacteria with the desirable characteristics is quite difficult. It is believed that more than 99% of microorganism have not been successfully cultivated yet and, surely, among this number many PHA-producing strains can be found (Wintermute and Silver, 2010). For most bacteria, the ability of accumulating internal reserves represents a major competitive advantage to survive in natural environments. Carbon-based internal reserves as PHA and glycogen or external like some polysaccharides, as well phosphorus (namely, polyphosphate – PolyP), nitrogen (cyanophycins) or sulfur-based reserves, are usually advantageous for the producing organisms and were found in many different species of bacteria, including those that constitute activated sludge populations, the most known and studied MMC. Activated sludge is a complex consortium of several populations of microorganisms of undefined composition, which depends on feeding and process conditions. Other MMC can be found in open biological systems that appear in natural environments, as soil, seawater, sediments, rumen, N_2-fixing bacteria, or, as activated sludge, in human created environments under non-sterile conditions, namely ponds, waste storage facilities, etc.

2.1.2. Historical background

Due to its role in wastewater treatment plants (WWTPs), the first MMC to be studied was activated sludge. In early 70s, the presence of PHA granules inside cells belonging to an activated sludge community was observed for the first time (Wallen and Rohwedder, 1974). Later, PHA were found to play a key-role on the competition between PolyP-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) for the external carbon source in enhanced biological phosphorus removal (EBPR). In this

type of systems, PHA are stored when the external carbon source, usually a SCOA, is supplied in the absence of an external electron sink. The lack of electron sink prevents microbial growth but PHA-storing bacteria are able to store the external carbon source as intracellular reserves to be used as carbon and energy source in the absence of external substrate and/or an electron sink is available (Pereira et al., 1996). In this way, PAOs and GAOs were the first organisms present in MMC to be described as PHA-storing bacteria. Later on, a correlation between PHA storage and use and the phenotypic identity of PAOs and GAOs was stablished by using fluorescence in situ hybridization (FISH) and postFISH nile blue staining (Levantesi et al., 2002). The first attempts to identify the microorganisms involved in EBPR, including both PAOs and GAOs, started almost 40 years ago, but so far no attempts were successful. Potential methods to successfully isolate these bacteria were tested through the use of e.g., micro-manipulation techniques from enriched cultures of these organisms (Oehmen et al., 2007). In the following years, PHA production by MMC systems based on the alternation of availability of electron sink was developed, being the most successful those where GAOs were dominant (Pisco et al., 2009).

Bulking was another aspect of activated sludge correlated with PHA storage observed in aerobic WWTP (Majone et al., 1996). The process configuration with alternation of excess of carbon source periods with depletion periods was observed to disfavor the selection of filamentous bacteria responsible for the bulking phenomena and select floc-formers with enhanced PHA storage capacity (Majone et al., 1996). The observations of Majone et al. (1996) confirmed the hypotheses raised by Daiger and Grady (1982) to explain the phenomenon. After a period of absence of external carbon source, during which the levels of enzymes and RNA required for growth decreased, a sudden increase of external substrate triggered the storage of internal reserves (faster response). Under such conditions, cell growth requires the synthesis of more enzymes and RNA which represents a more complex physiological adaptation resulting in a slower response (Daigger and Grady, 1982). This mechanism was the basis for the development of PHA production by MMC based on intermittent substrate supply. The concept of aerobic “feast and famine” process, later known as aerobic dynamic feeding (ADF), was

first proposed by Majone et al. (1996) and was widely used in the subsequent years (Albuquerque et al., 2007; Dionisi et al., 2004; Jiang et al., 2012; Lemos et al., 2006; Serafim et al., 2004; Villano et al., 2010a). Also the previously developed system based on the alternation of availability of oxygen could be considered a feast and famine process, since the external carbon source is available only during the absence of oxygen (Serafim et al., 2008a).

2.1.3. Mixed cultures versus pure cultures

In recent years, MMC processes gained marked importance and become competitive with pure cultures since they represent a clear alternative that can contribute to the decrease of PHA production costs. While pure culture processes, including those that employ recombinant organisms, rely on tight axenic conditions to guarantee the presence of the bacteria of interest, this is not an issue for processes using MMC. In MMC processes, the dominance of PHA-storing organisms is imposed by the operational conditions. The operational conditions chosen, usually based on the alternation of periods of excess and lack of carbon substrate, should allow only for the selection of PHA-storing organisms. This situation requires that the selected PHA-storing bacteria should consume completely the external substrate to establish true feast and famine conditions. The famine period is relevant since it allows eliminating the non-storing bacteria. Moreover, during this period, the PHA-storing bacteria will use their internal reserves as carbon and energy source, and the decrease of these reserves will trigger a new storage process when the next feast period begins.

The use of complex feedstock as substrates is another advantage of mixed cultures over pure cultures, since a wide range of low value substrates was already successfully tested, including industrial waste of undefined composition or containing known microbial inhibitors (Moita et al., 2014; Moita and Lemos, 2012; Palmqvist and Hahn-Hägerdal, 2000a, 2000b; Queirós et al., 2016). Also, the lack in SCOA of most of the feedstock, usually rich in carbohydrates, is not a drawback of MMC processes, since a preliminary acidification performed by anaerobic MMC can be included in the process, to convert the organic compounds into the desired substrates. The utilization of inexpensive

feedstock and the possibility to work without sterile conditions can contribute to the decrease of PHA production costs, when compared with pure culture processes.

Some weaknesses can be appointed to MMC processes, which are contributing to delay their implementation at industrial level. The first one is biomass concentration, which are way below than the values obtained in pure culture processes. The increase on the amount of biomass would improve volumetric productivities and may have a high impact on the downstream process costs, since extraction yield is directly dependent on the amount of biomass collected (Serafim et al., 2008a).

Another drawback traditionally appointed to MMC processes is the composition of the microbial population selected. A wide range of bacteria was already identified in MMC production systems as recently reviewed by Queirós et al. (2015), many of them traditional bacteria related with pure culture systems (Queirós et al., 2015). Moreover, the diversity of populations is related with robustness of the systems to cope with situation where the feedstock suffers periodic variations in its composition (Carvalho et al., 2014; Erik R. Coats et al., 2007; Dias et al., 2006; Pisco et al., 2009). The hypothesis that this diversity could reflect negatively on the PHA characteristics is raised quite often, despite some works already showed the opposite, by analyzing molecular weights, polydispersities, melting and glass transition temperatures, melting enthalpies or crystalinity (Laycock et al., 2013; Serafim et al., 2008b). More work in this area is still required since the characterization of the polymer produced is mandatory for the definition of the final application. Pure cultures allow for a better control of PHA characteristics and this is a requirement of polymer processing industries. Nevertheless, PHA monomeric composition can be controlled by feeding the MMC with defined mixtures of SCOA as already showed by several researchers (Albuquerque et al., 2011; Serafim et al., 2008b). Different mixtures of SCOA can result in the production of copolymers of poly-(3-hydrobutyrate-co-3-hydroxyvalerate) (P(3HB-*co*-3HV)) with different composition and also other monomers as 3-hydroxy-2-methylbutyrate (3H2MB), 3-hydroxy-2-methylvalerate (3H2MV) or 3-hydroxyhexanoate (3HHx) were already obtained in MMC processes (Pisco et al., 2009). The variety of monomers that can be obtained by MMC is very low when compared with pure cultures, since monomers from 3

to 14 carbons were already obtained as well with a high diversity of side groups (Chen and Hajnal, 2015; Steinbüchel and Steinbüchel, 1995; Tan et al., 2014).

The downstream processing is the one of the main drawbacks of PHA production by MMC. Despite the many methods tested (Madkour et al., 2013), including some of those working with pure cultures, the first positive results obtained were recently published (Jiang et al., 2015; Samorì et al., 2015; Villano et al., 2014). Cell wall and floc structure of MMC biomass seem to be much more complex and harder to disrupt than pure cultures that become fragile when high PHA storage contents are attained. Still, researchers are looking for a low-cost, highly efficient, and environmentally friendly PHA recovery process that allows for high purity without polymer degradation.

Since the MMC process is based on the use of industrial waste or subproducts and microbial populations collected in WWTP facilities, the possibility of implementing the PHA production units on those locations should be explored as a cost-effective technology. However, only a few pilot plants were already being tested in the last five years (Anterrieu et al., 2014; Janarthanan et al., 2016; Morgan-Sagastume et al., 2015; Ntaikou et al., 2014; Tamis et al., 2014) which is far from the situation of pure culture processes, already working at industrial scale. Moreover, the origin of PHA produced could raise some concerns about the final application of the polymer since some of the components of the feedstock used as substrate could contain impurities hard to eliminate from the extracted product. Also, the use of microbial populations selected in sewage treatment systems could be a problem for the commercialization. In these situations, the final application of the polymer cannot be the traditional applications of PHA produced by pure cultures, as medical field and food and cosmetics packaging and disposable items, but less noble applications in agriculture and packaging of non-food items.

The objective of this chapter is to exploit the different aspects related with PHA production by MMC.

2.2. Metabolism

Few specific works are available in the literature concerning the metabolism of PHA synthesis using MMC. For most of the cases, it is assumed that MMC perform in the same way as pure cultures. MMC preferred substrates for PHA production are SCOA, as acetic, propionic, butyric and valeric acids, resulting frequently in short-chain PHA.

Two types of mixed cultures present in activated sludge processes involving anaerobic/aerobic (AN/AE) steps were characterized in terms of PHA metabolism. PAOs are the responsible for phosphorus sequestration from wastewaters, being PHA important intermediates in this process. GAOs also strive in such systems differing from the previous organisms mainly in the form of obtaining energy, where polyP is not involved, competing for the same carbon substrates. Both uptake SCOA converting them to the respective hydroxyacyl-CoA and being polymerized to produce short-chain PHA. Under anaerobic conditions glycogen plays a major role both as provider of part of the energy and reducing equivalents used but also has a source of monomers for PHA synthesis, as acetyl or propionyl moieties (Lemos et al., 2007, 2003; Maurer et al., 1997; Pereira et al., 1996). Both polymers are interconnected since while under anaerobic conditions PHA are produced with glycogen consumption, under aerobic condition carbon contained in the previously produced PHA are converted to glycogen.

The most well studied pathways involve the production of poly-3-hydroxyalkanoates. The consumption of acetic acid to produce poly-3-hydroxybutyrate (P(3HB)) by *Cupriavidus necator* is the most common example. Here, acetic acid is converted to acetyl-CoA, two of such units condensed to acetoacetyl-CoA by the action of ß-ketothiolase, further processed to R-3-hydroxybutyryl-CoA (3HB) using acetoacetyl-CoA reductase, being this monomer added to the growing polymeric chain of PHB by PHA synthase. Other SCOA, as butyric and valeric acids, are also converted to their respective 3-hydroxyacyl-CoA (3HB and 3-hydroxyvaleryl-CoA (3HV), respectively). From propionic acid more than one monomer can be obtained, as combining two units of propionyl-CoA, 3H2MV can be obtained, while when one acetyl moiety combines with one propionyl moiety 3H2MB results.

The consumption of sugars and longer-chain organic acids can be performed in different ways. Sugars can be converted by glycolysis to pyruvate and further transformed into acetyl or propionyl moieties following the previously described scheme or the obtained acetyl-CoA introduced in the de novo fatty acids biosynthesis (Fig. 2.2). The R-3-hydroxyacyl-ACP intermediates of this pathway can be processed by a transacylase followed by a reductase to the corresponding R-3-hydroxyacyl-CoAs. Fatty acids can also be consumed in this pathway for monomer production in PHA synthesis but can likewise be processed through the catabolic fatty acids pathway, ß-oxidation. Concerning ß-oxidation, several of its intermediates can be drifted for R-3-hydroxyacyl-CoA production using enzymes as reductases, hydratases and epymerases. From these two substrates both SCL and MCL monomers can result.

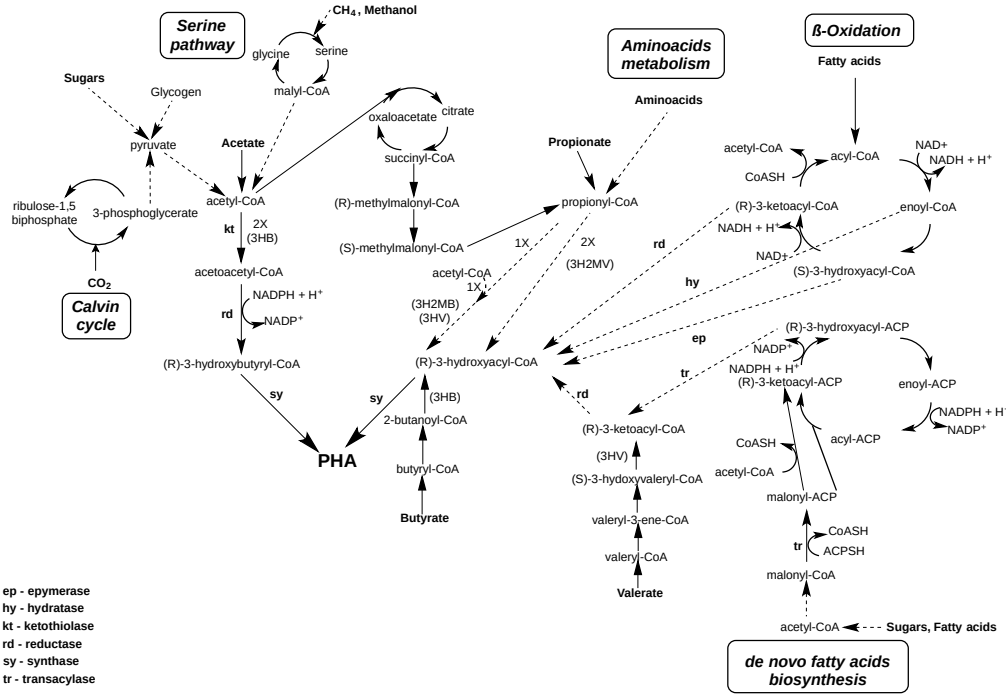

Fig. 2.2. Metabolic pathways for poly-3-hydroxyalkanoates production from different carbon sources (adapted from Serafim et al. (2008) and Tan et al. (2014)).

The type of PHA synthase of the microorganism determines the type of PHA produced, SCL- or MCL-PHA. So far, four different types of synthases are known, based on

the nucleotide sequences of 59 PHA synthase genes obtained from 44 different bacteria (Rehm, 2003). This classification is based on the substrate specificity and subunit structure. The type organisms for PHA synthases Class I, II, III and IV are *Cupriavidus necator*, *Pseudomonas aeruginosa*, *Allochromatium vinosum* and *Bacillus megaterium*, respectively. As for substrate specificity, Class I and III PHA synthases incorporate only short-chain-length hydroxyacyl monomers (C3–C5), while class II PHA synthases utilize medium-chain-length hydroxyacyl monomers (C6–C14). Class IV synthases are capable of polymerizing mainly short-chain-length monomer units but some works reported the ability of *B. megaterium* DSM 509, *Bacillus* sp. US163, and *Bacillus* sp. US177 to produce medium- or long-chain length PHA. Some diversity seems to exist among Class IV synthases since *B. cereus* and lineages have a broader substrate specificity producing PHA_{MCL} from a wider variety of monomers than the class representative *B. megaterium* and its lineages (Tsuge et al., 2015). Concerning the subunits structure, Clases I and II have only one subunit (PhaC) whereas class III is composed of two subunits, namely, a catalytic subunit PhaC and a second subunit PhaE. Class IV has also two subunits a catalytic subunit PhaC and a second subunit PhaR. Based on the differences on the nucleic acid sequences of class IV phaC Solaiman and Ashby (2005) suggested that this class could be divided into at least two subgroups one defined by *B. cereus* and another by *B. megaterium* (Solaiman and Ashby, 2005).

Other carbon compounds were also investigated as possible substrates for PHA production. Amino acids, as valine and isoleucine, were also shown to be involved in PHA production by a strain of *Alcaligenes eutrophus* (presently *Cupriavidus necator*) where the aminoacids provided the propionyl moiety present in the obtained copolymer of P(3HB-*co*-3HV) (Steinbüchel and Pieper, 1992). Substrates with a single carbon have also been considered, namely for their low cost, as carbon dioxide, methane and methanol. Hydrogen-oxidizing bacteria, as *Cupriavidus necator* and *Ideonella* sp., can utilize H_2 as electron donor and O_2 as electron acceptor to fix CO_2 and incorporate into bacterial biomass (Tanaka et al., 2011, 1995). These autotrophs recur to the enzyme RUBISCO present in the Calvin cycle from which 3-phosphoglycerate can be drifted for central metabolism via pyruvate, or to the utilization of the reverse tricarboxylic acid cycle,

providing acetyl-CoA moieties. Methane and methanol can be utilized by methylothophs for PHA production using either the serine pathway or the ribulose monophosphate (RuMP) pathway. The serine pathway is the most utilized for PHB production namely by *Methylocystis* and *Methylosinus*, *Methylococcus*, and *Methylomonas* spp (Khosravi-Darani et al., 2013). The utilization of CO_2 and CH_4 apart from a cheap substrate for PHA production could also help to mitigate the growing increase of greenhouse gases in the atmosphere.

Due to the high diversity of monomers, naturally occurring or chemically synthetized, PHA with monomers other than 3-hydroxyalkanoates can be produced. The presence of 4-hydroxybutyrate monomers (4HB) in PHA, with high utilization on biomedical applications, can be obtained from the use of 4-hydroxybutyric acid, γ-butyrolactone, 1,4-butanediol and other α, ω-alkanediols as carbon sources (Doi et al., 1990). Other examples are the utilization of cyclohexanol to the production of 6-hydroxyhexanoyl-CoA and of 4,5-alkanolactone to obtain 4,5-hydroxyacyl-CoA (4,5-HA-CoA) (Chen, 2010).

2.3. Strategies of PHA production by MMC

In the recent years, numerous approaches were studied to produce PHA, making use of a variety of organisms such as bacteria, plants and fungi. Also, with the advent of genetic engineering, the use of several recombinant organisms become possible, like the well-known recombinant *Escherichia coli* or new transgenic plants (Koller et al., 2011). With the aim of achieving the industrial production, several considerations can be considered: choosing cheap and suitable substrates in accordance with the metabolism of the producing organism; its storage capacity; the need for sterile conditions; type of reactor, feeding pattern; oxygen demand; behavior and evolution of the culture.

Over than 300 bacterial species were reported as PHA producers but only 75 were tested for large-scale production. Currently, industrial production processes rely on pure cultures of microorganisms in their wild form, or genetically modified, namely, *Cupriavidus necator*, *Alcaligenes latus*, *Comamonas acidovorans*, *Pseudomonas putida*, *Chromobacterium* sp., *Hydrogenophaga pseudoflava*, *Rhodococcus rubber*, *Azotobacter*

vinelandii, *Pseudomonas oleovorans*, *Paracoccus denitrificans*, *Protomonas extorquens* and recombinant *Escherichia coli* (Magdouli et al., 2015). Genetic engineering allowed for the construction of recombinant strains for a more cost-effective PHA production. By cloning PHA synthesis genes from several organisms, strains showing simultaneously fast growth and high cell density from inexpensive substrates and simple polymer purification, can be obtained. However, the use of pure culture and genetically modified organisms still represent high production costs and, as mentioned before, one way to overcome them is the use of MMC.

2.3.1. First attempts

In PHA production by MMC, the selection of strains with high capacity to accumulate intracellular reserves results from the imposition of transient conditions. The production of PHA by MMC was first verified in the EBPRs facilities (Serafim et al., 2008a). Activated sludge processes with alternating anaerobic and aerobic conditions have been successfully used for EBPR from wastewater. Such EBPR processes are referred to as the AN/AE or anaerobic-oxic (AN/AO) process (Mino, 2000). This process comprises two distinct phases. The anaerobic stage in which absolute anaerobic conditions are kept with neither oxygen nor NO_2^-/NO_3^- available as electron acceptor. During this stage, the sludge only has at its disposal organic substrates from the phosphorus- enriched influent wastewater. Prompt uptake of organic substrates by PAOs is usually accompanied by phosphorus release, from stored PolyP, to the bulk medium. Energy from this hydrolysis is used to build PHA as a consequence of oxygen limitation in the presence of external organic carbon (Fig. 2.3). A second carbon-based storage reserve, glycogen, plays also a role by acting as an electron sink in the absence of an external one. The second stage is characterized by the presence of an external donor acceptor, oxygen or nitrate, and PAOs can utilize the stored PHA to grow and restore glycogen reserves and replenish PolyP from the soluble phosphate of wastewater. Depending on the conditions and substrates in such systems, also GAOs can be enriched, which are also able to produce PHA but compete for carbon sources with PAOs. During the aerobic/anoxic stage, PAOs uptake more Pi than the released in the previous anaerobic stage (Oehmen et al., 2007).

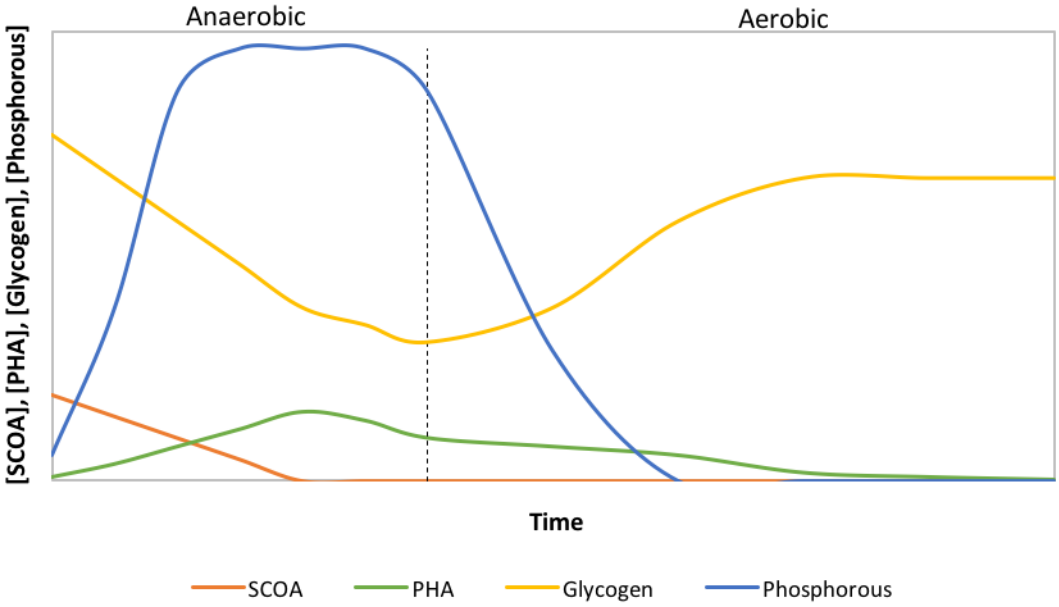

Fig. 2.3. Example of an anaerobic/aerobic cycle operation by PAOs.

In contrast, several low/zero Pi-content waste- and by-products can be used to enrich a culture in more efficient PHA-storing microorganisms. Depriving sludge from Pi, the community will channel the organic compounds towards PHA accumulation through glycogen consumption, maximizing its content to survive the aerobic stage without external substrate available (Fig. 2.4). For this reason, GAOs gained importance as PHA-storing organisms in MMC processes (Bengtsson, 2009; Pisco et al., 2009). Nevertheless, the amount of PHA accumulated by these groups of microorganisms is generally lower than those selected by ADF further exploit in the next section (Dias et al., 2006).

Another process for PHA production is the microaerophilic-aerobic system, which is a modification of the anaerobic-aerobic one. In this process, an aerobic period alternates with microaerophilic conditions, where a very limited amount of oxygen is supplied. In this system, microorganisms consume organic substrates that allow for the achievement of energy through oxidative degradation. If the supply of oxygen is sufficient, microorganisms are able to get enough energy for assimilative activities such as the production of protein, glycogen, along with consuming of organic substrates. If the supply of oxygen is adequately controlled, the assimilative activity will be suppressed and

the external substrate is drifted to PHA accumulation (Satoh et al., 1998). As PHA production requires less energy than needed for glycogen synthesis, the oxygen supplied should be around 0.51% of the chemical oxygen demand (COD) provided, to allow bacteria to store high amounts of PHA intracellularly (Satoh et al., 1998). Pratt et al. (2012) also observed that a higher fraction of substrate was accumulated as PHA in comparison to high dissolved oxygen (DO) conditions. However, the time required to accumulate the same PHA amount as in high DO conditions was three times longer (Pratt et al., 2012).

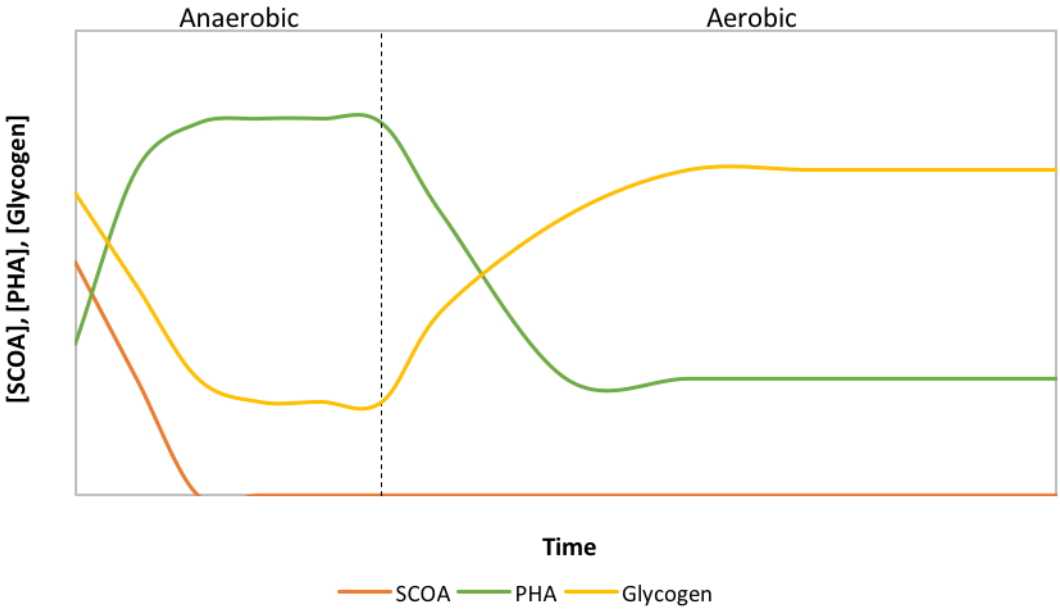

Fig. 2.4. Example of an anaerobic/aerobic cycle operation by GAOs.

An alternative to both processes described is the ADF system, first proposed by Majone et al. (1996). In this process, also known as "feast and famine", the MMC, under aerobic conditions, is submitted to alternating periods of external substrate availability (feast) and unavailability (famine) and results in unbalanced growth. The absence of a carbon source during a period of time causes a decrease in the number of intracellular components fundamental for the cell growth (RNA and enzymes). Following this period of famine, if the MMC is fed with an excess of carbon, as the number of enzymes available is

lower than the necessary for a maximum growth rate, the storage of PHA becomes the dominant phenomena. The ADF process will be described in more detail in the next sub-chapter, since it is the most studied and developed in the last years.

2.3.2. 3-Step Process

To become economically competitive with pure cultures, low-value substrates, such as agro-forestry residues, industrial by-products, and industrial and urban waste, can be repurposed for PHA production by MMC. Nonetheless, these low-value substrates need to be converted into readily biodegradable carbon sources to be transformed into PHA by microorganisms in MMC. In 2004, Dionisi et al. proposed a three-step process for using waste or surplus-based feedstock: (1) Acidification of organic components of the feedstock to obtain readily biodegradable carbon sources, as SCOA; (2) Enrichment in PHA-storing microorganisms; (3) PHA accumulation (Fig. 2.5) (Dionisi et al., 2004).

The acidification step is required whenever the chosen feedstock is poor in SCOA. This is a stage of anaerobic digestion, where carbon-based compounds from waste are transformed into SCOA (e.g., acetic, propionic or lactic acids) and alcohols (e.g., ethanol). The anaerobic digestion is a complex process, which can be divided into four individual degradation stages: hydrolysis, acidogenesis, acetogenesis, and, finally, methanogenesis. Anaerobic digestion is a treatment-oriented technology employed within full-scale facilities worldwide for the treatment of industrial and urban wastewater and organic solid waste. This process is advantageous over aerobic systems because of high organic removal rates, low energy-input requirement, energy production, and low sludge production. However, for SCOA production, it is essential to eliminate methanogenesis by manipulating parameters such pH, temperature, hydraulic retention time (HRT), sludge retention time (SRT), and organic loading rate (OLR). Therefore, the amount and type of SCOA generated by the fermentation of organic constituents are crucial for the selection and accumulation steps, influencing the amount and type of PHA produced (Albuquerque et al., 2011, 2010b; Lemos et al., 2008; Serafim et al., 2008b, 2006).

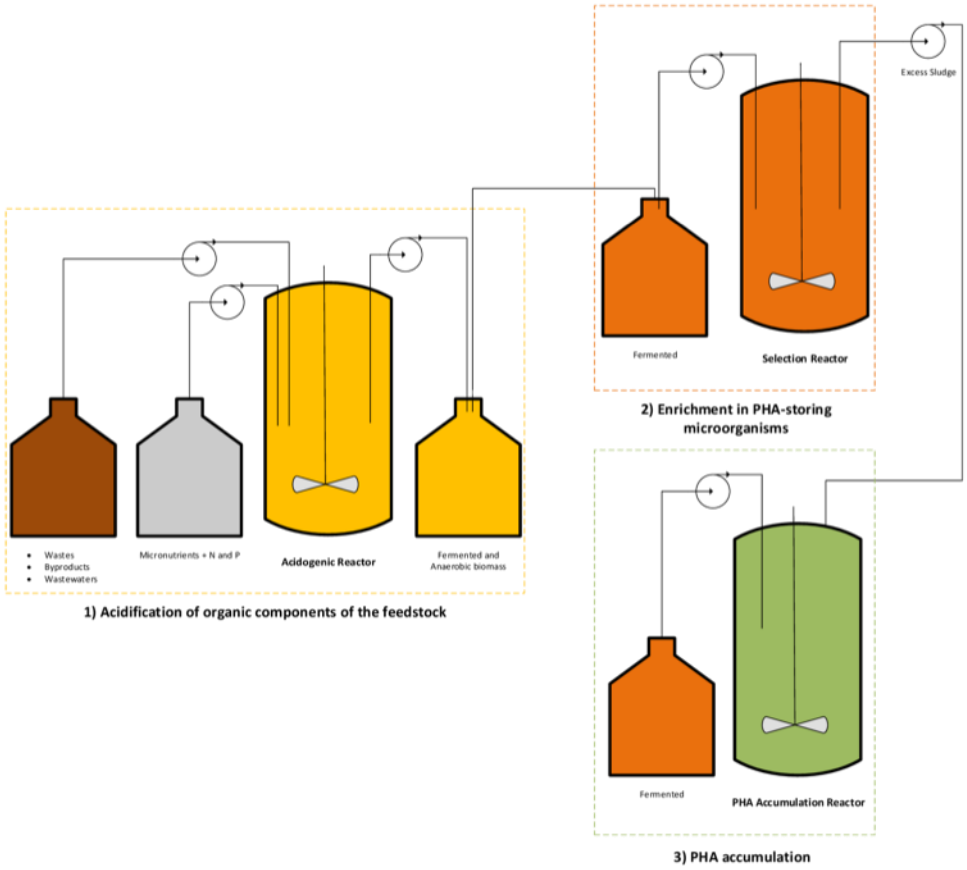

Fig. 2.5. 3-step PHA production process (Adapted from Dionisi et al. (2004)).

The enrichment in PHA-storing organisms is considered one of the key-points of all PHA production process by MMC. The objective of this step is to select the maximum number of organisms from the initial culture, with a high PHA storage capacity and eliminate, or at least reduce, non PHA-storing side populations. The system is operated to eliminate microorganisms presenting low or no storage capacity since they contribute negatively for the reduction of the average PHA cell content and storage yields by using the carbon source for growth. Very heterogeneous populations in terms of PHA storage ability have a negative impact on the downstream process. However, experimental results showed that by keeping a high storage capacity at this phase an unstable population is usually obtained. Thus, the operation of the selection reactor should be optimized to obtain a homogeneous population with relatively high and stable storage capacity rather than to maximize the PHA cell content (Serafim et al., 2008a). The

accumulation step is usually projected to maximize the content of polymer stored by cells harvested from the selection step using the substrate obtained from the acidification step.

In the 3-step process, ADF is the most common methodology applied for biomass selection. In ADF systems, the availability of an excess of external substrate after a long period of absence triggers the accumulation of intracellular reserves, due to the lower requirements of physiological adaptation by microorganisms (Fig. 2.6). In this situation, the substrate is used almost entirely to the accumulation of intracellular reserves (about 70% of the substrate). However, cell growth can become dominant if the exposure time to substrate is long enough to allow physiological adaptation of microorganisms (Majone et al., 1999; van Loosdrecht et al., 1997). Once more, parameters such pH, temperature, HRT, SRT, and OLR, among others, are the key to successfully steer the selection (Reis et al., 2011). Despite the use of synthetic substrate, Johnson et al. (2009) selected a stable microbial community able to reach 89% of cell dry weight (cdw) in the accumulation step, a value comparable to those obtained with pure cultures in terms of maximum PHA content (Johnson et al., 2009). Molecular techniques confirmed that the operational parameters imposed led to a virtual monoculture, highly competitive, later fully characterized and named *Plasticicumulans acidivorans* (Jiang et al., 2011).

A considerable number of works already began seeking for processes based on using complex substrates, namely olive mill wastewaters (Dionisi et al., 2005b), paper mill wastewaters (Bengtsson et al., 2008b; Jiang et al., 2012), pulp and paper mill by-products (Queirós et al., 2014), palm oil mill effluents (Mohd et al., 2012), pyrolysis by-product and crude glycerol (Moita et al., 2014; Moita and Lemos, 2012) and sugar cane molasses (Albuquerque et al., 2007). Using fermented paper mill wastewaters, Jiang et al. (2012) obtained an MMC able to accumulate the highest PHA content so far reported with a complex substrate, namely 77% of cdw (Jiang et al., 2012).

The mechanisms for PHA storage by PAOs and GAOs enriched under anaerobic/aerobic conditions are different from those observed in the ADF process. Nevertheless, in both cases, PHA storage occurs when growth is prevented. Contrary to ADF, where PHA storage occurs due to an internal growth limitation despite the presence

of electron donor and acceptor, in the AN/AE process, storage is mainly caused by an external growth limitation due to absence of an electron sink (oxygen and/or nitrate). A few works employed this selection strategy, aiming the enrichment in GAO phenotypic behavior, reaching however lower PHA accumulations (Bengtsson, 2009; Liu et al., 2013; Pisco et al., 2009).

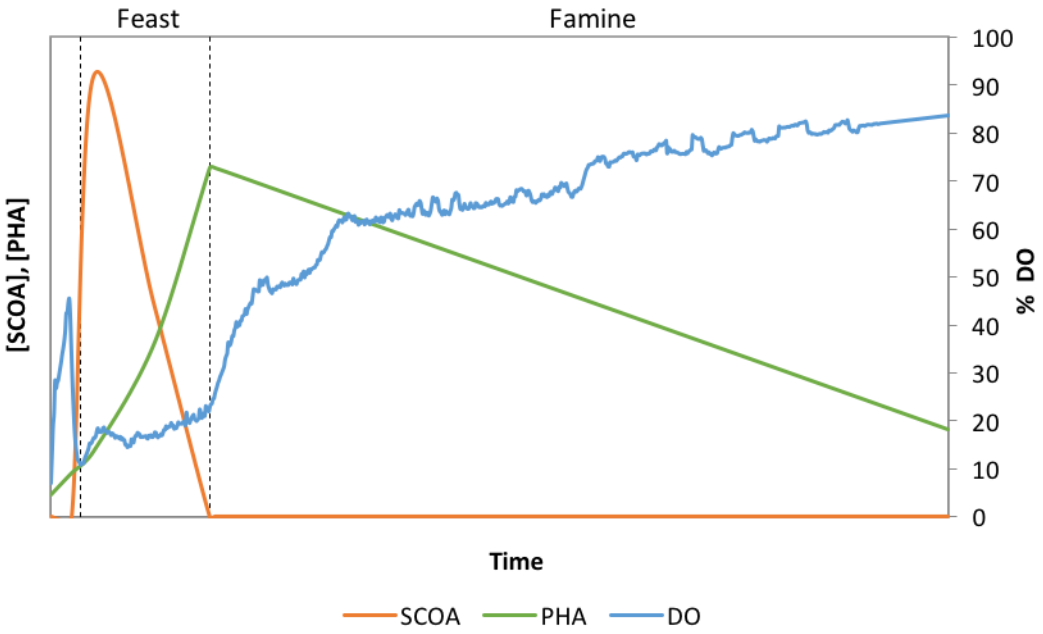

Fig. 2.6. Example of an ADF cycle operation by a fully aerobic culture.

High accumulations are obtained at the third and last step of the process. The excess sludge produced in the selection reactor with a high storage response is exploited in a third reactor, operated at considerably higher organic load to saturate the sludge storage capacity. The accumulation stage, thus, aims the PHA accumulation in microbial cells and its efficiency strongly depends on the storage capacity of the selected MMC. A highly relevant parameter is the maximum attainable polymer content in the biomass that can decrease the polymer extraction cost. If the accumulation is performed under nutrient-limiting conditions the driving force for storage will be kept high until a PHA saturation level is reached. On the contrary, if the biomass is continuously exposed to nutrient rich conditions plus high organic loads, a growth response will progressively take

place, overcoming, eventually, the storage response. Therefore, the maximum PHA content will be lower than the maximum storage capacity.

Finally, the PHA-rich biomass then flows to the downstream processing for extraction and purification of PHA.

2.3.3. Operation Modes

Selecting a culture with a high storage capacity is one of key stones in MMC processes to develop an economical and competitive PHA production process. The goal is to steer the population to a homogeneous microbial enrichment in terms of high PHA storage capacity (Dias et al., 2006; Queirós et al., 2015). Selections leading to heterogeneous communities, in terms of PHA accumulation, will terminate into a negative impact on the overall productivity due to a reduction on the average PHA cell content, and feasibility of the process by increasing the PHA extraction costs. Nevertheless, the selection step should aim for a homogenous population with stable storage capacity, leaving the maximization of PHA cell content for the accumulation step (Dias et al., 2006).

During the selection period, the population shifts and dynamics should be followed to correlate the communities selected with the performance and results observed (Queirós et al., 2015). Several molecular methods, such FISH, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), pyrosequencing, and quantitative-PCR (qPCR) allow monitoring periodically the microbial community evolution, identifying the different groups of microorganisms and handle the selection towards the best PHA producers (Queirós et al., 2015).

Several strategies can be implemented to select PHA-storing microorganisms, from which stand out the AN/AE and ADF systems.

2.3.3.1. Anaerobic/Aerobic System

One of the main sources of pollution is the one caused by phosphorous accumulation due to industrial and agriculture intensive activity. To prevent a drastic decrease in water quality and aquatic ecosystems losses, EBPR systems were created and

applied (Ramasahayam et al., 2014). Based on this method, anaerobic/aerobic system was created. In this process, as mentioned before, PAOs and GAOs showed to be able to accumulate PHA (Serafim et al., 2008a).

A considerable number of works considered the use of synthetic mediums/synthetic wastewaters to study the selection of PHA-storing microorganisms under AN/AE conditions and understand its influence on MMC enrichment (Table 2.3). Michinaka et al. (2007) aimed to select and identified the bacteria able to accumulate PHA containing 3H2MV by an MMC selected in an AN/AE SBR with synthetic wastewater containing propionic acid: acetic acid (14:3) as carbon sources. In fact, authors observed an evolution towards the increase on the amount of 3H2MV, from 6 to 38%. Through the construction of *phaC* clone libraries, 261 clones were found to have a putative lipase box, a conserved block, probably to be the catalyst center of PHA synthase (Rehm, 2003), and applying T-RLPF the authors related the increase amount of 3H2MV produced with the presence of *Pseudomonas* sp. (Michinaka et al., 2007). Before moving towards complex substrates, synthetic ones were explored to select the best operational parameters. For 450 days, a stable reactor submitted to AN/AE was operated using only acetic acid (Bengtsson, 2009). The GAOs enriched culture accumulate up to 49% cdw (3HB:3HV = 73:27) under anaerobic condition and up to 60% P(3HB) of cdw of under aerobic condition, in batch tests. Regarding microbial characterization *Candidatus* Competibacter phosphatis accounted to 57% and *Defluviicoccus*-related Cluster 1 and 2 – 6% during the steady-state, both communities undetected in the beginning of the experiment (Bengtsson, 2009). Other parameters such the effect of COD/N ratio, were also studied using an AN/AE SBR fed with acetic acid. COD amount was gradual increased up to 1200 mg L^{-1} and COD/N ratio fixed at 125, culminating in a PHA accumulation of 64.2% of cdw (Liu et al., 2013).

To follow the evolution, pyrosequencing allowed to identified a very rich community of nine phyla: *Proteobacteria*, *Acidobacteria*, *Firmicutes*, *Bacteroidetes*, *Gemmatimonadetes*, Cyanobacteria/Chloroplast, *Actinobacteria*, *Nitrospira*, TM7, and unclassified bacteria, representing approximately 36.34, 14.09, 4.41, 1.72, 0.22, 1.51, 19.68, 0.11, 0.75, and 21.18% of the sequences, respectively (Liu et al., 2013). It was

demonstrated that microbial communities selected could be quite diverse and still present considerable PHA accumulation capacities (92 taxa of PHA-accumulator bacteria were presented by the authors).

Under AN/AE conditions and using hydrolyzed molasses, supplemented with acetic acid, as substrate *Pseudomonas* was selected as the main PHA-storing population. The MMC was able to accumulate up to 24.8% cdw PHA after 30 minutes of beginning of anaerobic phase (Ciesielski et al., 2008). Simple variations such the origin of the inoculum and the matrix of the feed can lead to the selection of different microorganisms.

The final objective, after understanding how the several operational parameters could influence and guide the culture selection, is the use and valorization of residues and by-products from several industries. Several complex substrates were already tested to select a PHA-storing culture. Operated under AN/AE conditions, condensate wastewaters from pulp and paper mill and fermented municipal primary solids were proven to be adequate substrates for PHA production. The reactor fed with condensate wastewaters from pulp and paper mill was operated for four months with 24 h cycles and an SRT of 4 days, showing an average P(3HB) production of 6.6% of cdw (Erik R. Coats et al., 2007). The SBR fed with fermented municipal primary solids was operated under AN/AE conditions for nine months, with a PHA content fluctuating between 10 and 25% cdw (Erik R Coats et al., 2007).

Despite the long-term selection, both reactors recorded low PHA storing values and considerable fluctuations. Lack of oxygen to nearly absence could have led to complete inhibition of microbial community, once it was necessary to degrade some complex carbon sources in the condensate (Erik R. Coats et al., 2007). Fermented paper mill wastewater was used, alongside with anaerobic/aerobic conditions, to select GAOs for PHA production. The SCOA-rich led to a population rich in GAOs related to *Defluviicoccus vanus* (56%) and *Candidatus* Competibacter phosphatis (22%). Further accumulation tests, by applying a subsequent anaerobic period after an initial aerobic, led to a PHA content of 42% of cdw (Bengtsson et al., 2008c). One last example falls on the use of food residues, such fermented sugar cane molasses. A culture already enriched in GAOs was submitted AN/AE conditions reached 17% in cdw of PHA, being composed by

3HB, 3HV monomers in the first 81 days of reactor operation (Pisco et al., 2009). The microbial community maintained the GAO-phenotypic behavior and bared the capacity on handling short-term variations in the feeding composition, consequence of the acidogenic fermentation. Given the feed composition, an unforeseen PHA_{MCL} monomer of 3HHx, confirmed by ^{13}C-NMR, was observed further on reactor operation (137 days operation time) (Pisco et al., 2009). Microbial characterization by FISH analysis assigned the appearance of 3HHx to a shift in the community of GAO species, from *Candidatus* Competibacter phosphatis to *Defluviicoccus vanus*-related organisms (Pisco et al., 2009).

Table 2.3. PHA-storing microorganisms selected under AN/AE conditions.

HRT	SRT	Substrate	Molecular Method	Main Organisms Selected	Study
8 h	10 days	Mixture of acetic and propionic acid	T-RFLP	*Pseudomonas sp.*	Michinaka et al. (2007)
1.3 days	10 days	Acetic acid	FISH	*Candidatus* Competibacter phosphatis, *Defluviicoccus*-related Cluster 1 and 2	Bengtsson (2009)
18 h	7 days	Synthetic wastewater	Pyrosequencing	*Proteobacteria*, *Acidobacteria* *	Liu et al. (2013)
2 days	4 days	Hydrolyzed molasses supplemented with acetic acid	DGGE	*Pseudomonas*	Ciesielski et al. (2008)
3 days	10 days	Fermented paper mill wastewater	FISH	*Defluviicoccus vanus*, *Candidatus* Competibacter phosphatis	(Bengtsson et al. (2008c)
1.33 days	10 days	Fermented sugar cane molasses	FISH	*Defluvicoccus vanus-related organisms*	Pisco et al. (2009)

* - 92 different taxa were identified

2.3.3.2. Aerobic Dynamic Feeding System

As previously stated, the most well studied process for the selection step is ADF. ADF process imposes, periodically, alternated availability of substrate creating "feast" and "famine" phases (Fig. 2.6).

Since its introduction by Majone et al. (1996), ADF became the best studied PHA storage process (Table 2.4). Similar to AN/AE process, the selection of PHA-accumulating organisms under ADF conditions was performed with synthetic media containing single or mixed organic acids as sole carbon sources. Several reactor operational conditions were tested: OLR, HRT, SRT, carbon to nitrogen ratio (C/N), pH, oxygen concentration and temperature (Dias et al., 2006). This was followed by the introduction of complex and low value substrates, such as agro-forestry residues, industrial byproducts and industrial and urban waste (Albuquerque et al., 2007; Bengtsson et al., 2008b; Erik R. Coats et al., 2007; Dionisi et al., 2005b; Moita and Lemos, 2012; Queirós et al., 2014). Dionisi et al. (2005) studied the selection of a PHA-storing community feeding a SBR with a mixture of SCOA, namely acetic, lactic and propionic acids. A high OLR was used as selective pressure, 12.75 gCOD L^{-1}, that led to an accumulation of 14.7% on COD basis (Dionisi et al., 2005a). DGGE revealed a significant shift on the predominant microbial population throughout the reactor operation time. Authors observed a continuous narrowing on the number of microbial populations from the initial activated sludge inoculum. From the DGGE bands sequencing, genus such *Methylobacteriaceae*, *Flavobacterium* and *Thauera* were identified, all PHA accumulators (Dionisi et al., 2005a).

Acetic and propionic acids are two of the most used SCOA in culture selection. To evaluate the culture behavior in the presence of these SCOA, two SBRs under ADF conditions, one fed with acetic acid (60 Cmmol L^{-1} day^{-1}) and the other with propionic acid (30 Cmmol L^{-1} day^{-1}), with 1 and 10 days of HRT and SRT and inoculated with an MMC from a stable phosphorus removal system, were studied (Serafim et al., 2006). The inoculum was dominated by *Candidatus* Accumulibacter phosphatis, *Candidatus* Competibacter phosphatis and tetrad-forming organisms (TFOs) affiliated to the *Alphaproteobacteria* class. After 5 SRTs, PAOs and GAOs disappeared because of the change from AN/AE to ADF conditions (Serafim et al., 2006). Despite the different feeding

and OLR, the microbial populations selected were similar between both SBRs, presenting, however, different metabolism that resulted in diverse polymer compositions. The MMC from the acetic acid fed reactor produced a homopolymer of P(3HB) and that from the propionic acid fed reactor, a copolymer of P(3HB-*co*-3HV) (3HB:3HV = 28:72) (Serafim et al., 2006). To understand the microbial changes operated in the reactors under the influence of the applied SRT and OLR, the population was quantified by FISH (Lemos et al., 2008). In reactor fed with acetic acid, 41.1 ± 2.2% of bacterial community belonged to *Thauera*, whereas it was only present at 1.9 ± 0.2% in the reactor fed with propionic acid. *Azoarcus* (23.3 ± 1.5%) and *Amaricoccus* (28.8 ± 1.8%) were the two main others genus present in the reactor fed with acetic acid. For the reactor fed with propionic acid, *Amaricoccus* was the dominant genus, 61.4 ± 1.9%, followed by *Azoarcus*, 3.9 ± 0.3%. A third SBR was introduced and operated under ADF conditions. Decreasing the SRT to 1 day and increasing the OLR of 120 Cmmol L^{-1} day^{-1} (acetic acid), faster-growing organisms were selected. In this way, *Amaricoccus* was washed out from the system and *Azoarcus* and *Thauera* accounted for 45.9 ± 1.5% and 49.4 ± 1.4%, respectively. This, consequently, also led to lower accumulations (Lemos et al., 2008).

By using synthetic medium with acetic acid as sole carbon source the MMC that stored the highest amount of PHA accumulation, so far recorded, namely 89% of cdw, was selected. This content was obtained by submitting the MMC to a fed-batch experiment under growth limiting conditions. Through standard DGGE and 16S rRNA clone library construction, it was verified that the operational parameters imposed led to a virtual monoculture, highly competitive, without the need for sterilization (Johnson et al., 2009). The organism was later fully characterized and named *Plasticicumulans acidivorans* (Jiang et al., 2011).

PHA can present several properties, manipulating its monomers constitution and side chains. PHA_{MCL} displays particular physical properties making them more suitable for several applications. An MMC was enriched in *Pseudomonas aeruginosa* by feeding an ADF SBR nonanoic acid, as explained in the metabolism sub-chapter (Lee et al., 2011). The culture was able to accumulate up to 8.9% of PHA in cdw at the end of the feast phase, with a monomeric composition of 3-hydroxynonanoate (3HN) and 3-hydroxyheptanoate

(3HHp) (79–84 mol-%, respectively) (Lee et al., 2011). Regarding the monomeric constitution, a strategy that relies on feed composition must be adopted. Some experiments revealed that for obtaining the desired 3HV fraction in P(3HB-*co*-3HV), a proper selection of carbon sources for sludge acclimation and substrate composition for the accumulation step was necessary (Wang et al., 2013). Therefore, the highest P(3HB-*co*-3HV) content with MMC was obtained from a culture acclimated to acetic and propionic acids as substrate and further fed, in a batch test, with a mixture of acetic propionic acids in a ratio of 1:1 (mg/mg). The MMC presented a P(3HB-*co*-3HV) content of 47.7% of cdw, with an 3HV fraction of 48 mol-% (Wang et al., 2013).

The DGGE applied to the culture showed that *Sphingomonadales*, *Rhizobiales*, *Rhodobacterales*, *Rhodocyclales*, *Actinomycetales* and *Sphingobacteriales* dominated in both acclimation conditions, with acetic and propionic acids, which permitted to conclude that the addition of propionic acid for acclimatization did not dramatically shift the composition of the microbial community (Wang et al., 2013). It was interesting to observe the disappearance of genera like *Thauera*, *Sphingomonas* and *Novosphingomonas*, selected at reactor fed with only acetic acid, with the introduction of propionic acid in the batch tests. The same was observed for *Flavobacterium* and *Diaphobacter*, although in this case the MMC was submitted to acclimatization with and propionic acids. *Acidobacteria* and *Burkholderiales*, microorganisms that can consume propionic acid, were dominant when the highest P(3HB-*co*-3HV) content was obtained. MMC, withdrawn from reactor fed with acetic and propionic acids, had a preference for propionic acid as substrate and utilized propionic before acetic acid, and the enrichment of these microorganisms may prompt P(3HB-*co*-3HV) accumulation (Wang et al., 2013).

Table 2.4. PHA-storing microorganisms selected under ADF conditions.

HRT	SRT	Substrate	Molecular Method	Main microorganisms Selected	Study
1 day	1 day	Mixture of acetic, lactic and propionic acids	DGGE	*Methylobacteriaceae, Flavobacterium*, *Thauera*	Dionisi et al. (2005a)
1 day	10 days	Acetic acid	FISH	*Thauera*, *Azoarcus*, *Amaricoccus*	Lemos et al. (2008); Serafim et al. (2006)
1 day	10 days	Propionic acid		*Azoarcus*, *Amaricoccus*	
1 day	1 day	Acetic acid		*Thauera*, *Azoarcus*	
1 day	1 day	Acetic acid	DGGE and 16S rRNA gene clone library	*Plasticicumulans acidivorans*	Jiang et al. (2011); Johnson et al. (2009)
3 days	3 days	Nonanoic acid	DGGE	*Pseudomonas aeruginosa*	Lee et al. (2011)
1.67 days	5 days	Acetic acid	DGGE	*Dechloromonas, Thauera,* Compectibacter lineage	Liu et al. (2011)
1.67 days	5 days	Acetic acid Acetic:Propionic acid (3:1)	DGGE	*Sphingomonadales*, *Rhizobiales*, *Rhodobacterales*, *Rhodocyclales*, *Actinomycetales*, *Sphingobacteriales*	Wang et al. (2013)
1 day	1 day	Mixture of acetic and propionic acids	DGGE	*Lampropedia hyaline*	Valentino et al. (2014)
1 day	10 days	Fermented sugar molasses	FISH	*Azoarcus, Thauera*	Albuquerque et al. (2010b)
1 day	10 days	Bio-oil from chicken beds fast-pyrolysis	FISH	*Thauera*, *Amaricoccus*, *Zoogloea*	Moita and Lemos (2012)
2 days	2 days	Fermented paper mill wastewater	FISH	*Plasticicumulans acidivorans*, *Thauera*	Jiang et al. (2012)
1 day	5 days	Hardwood sulfite spent liquor	FISH	*Alphaproteobacteria*	Queirós et al. (2014)

Besides feed composition, it is also important to explore the best operational parameters to select a culture. *Lampropedia hyaline* was the main microorganisms selected, that overcome the microbial population leading, thus, to the best results. In a reactor fed with a mixture of acetic and propionic acid, the OLR and HRT (Villano et al., 2010b), the pH-value and SCOA mixture (Villano et al., 2010a) and cycle length (Valentino et al., 2014) were optimized. In this way, and applying ADF regimen, an OLR of 8.5 gCOD L^{-1} and a pH-value of 7.5 were found to be the best conditions that led to higher PHA accumulations. DGGE analysis of 16S rRNA genes was done for every condition tested and revealed an enriched community dominated by *Lampropedia hyaline*. The best yields were observed with culture dominated by *L. hyaline* in opposition to a more heterogeneous culture which resulted in lower values of PHA production. Using the same OLR and pH value studied for a cycle length of 2, 6 and 8 hours it was observed a clear evolution of the culture for the different cycles. However, *Lampropedia hyaline* outcompeted the remain population for the 6 and 2 hours cycles leading to accumulation up to 50% gCODPHA $gCODVSS^{-1}$ in batch tests (Valentino et al., 2014). These findings proved that pH, OLR and cycle length play important roles in selecting the best PHA-storing community.

It is important to retain that despite the selection being, probably, the key factor in the PHA production by an MMC, not always a very narrow population is obtained specially with the use of complex substrates. In a different work, the enrichment and accumulation step led to an MMC enriched in PHA-accumulating organisms comprised in the *Dechloromonas*, *Thauera* and Compectibacter lineage (Liu et al., 2011). It is important to stress that these results showed that different process operating conditions, mainly those applied and studied in the batch testes, led to different microbial community structures, which can be linked to different metabolic pathways activated under different operating conditions. A final analysis was done with nonmetric multidimensional scaling, which proved that microbial community structure has a close relationship with the process operating conditions also applied (Liu et al., 2011). DGGE was applied to the original, acclimatized and PHB-accumulating MMC. Contrary to what was described so far, it was observed that the number of DGGE bands from the acclimated sludge samples

before or after P(3HB) accumulation increased significantly in comparison to the non-acclimatized sludge sample (Liu et al., 2011).

Besides attempting to reduce PHA production costs, another very significant advantage of this strategy comes up, that fits in the concept of biorefinery: the valorization of industrial residues with high organic load and low value that generally represent a disposal problem to the industry, as stated earlier.

Fermented sugar molasses was proven to be a suitable substrate to enrich an MMC in PHA-storing microorganisms. Albuquerque et al. (2007) operated a SBR without pH control, 12 h cycle, SRT of 10 days, with temperature control at 25 °C, ammonia limiting conditions and two different OLR (120 and 60 Cmmol L^{-1} d^{-1}). A stable PHA-accumulating culture was obtained when the lower OLR and higher ammonia concentration (2.5 mmolN L^{-1}) were used, accumulating up to 10% cdw PHA. It was also proven, this time for a complex substrate, that OLR and feast/famine ratio were a critical factor of the culture enrichment step, with the culture losing the PHA storage capacity at higher OLR for the same cycle length (Albuquerque et al., 2007). Later, Albuquerque et al. 2010 demonstrated that the SCOA profiles on fermented sugar molasses and feeding regimen allowed to manipulated the PHA composition (Albuquerque et al., 2011).

Food processing wastewaters can also be a potential substrate to be explored in PHA production. In 2008, the possibility of using tomato cannery waste for simultaneous wastewater treatment and selection of PHA-storing microorganisms under ADF conditions was studied (Liu et al., 2008). Authors adopted a two-stage process, where the selection bioreactor was operated with 4 days as HRT and SRT. The selected populations were able to remove 84% of the present COD and accumulate PHA within the range of 7% to 11% cdw in non-filtered wastewater and of 2% to 8% in filtered wastewater. A maximum PHA content of 20% of cdw was obtained in batch tests (Liu et al., 2008). Moita and Lemos (2012) used bio-oil resulting from the fast-pyrolysis of chicken beds to acclimatize an MMC to produce PHA. An ADF system SBR, with 12 hours cycle, was operated with a HRT of 1 and an SRT of 10 days. It took 167 days to reach a pseudo-steady state where the sludge could accumulate up to 9% cdw of P(3HB-*co*-3HV) with 30% of 3HV. Glycogen was also stored, 2.9% of cdw, probably due to the presence of

sugars in bio-oil (Moita and Lemos, 2012), decreasing the volumetric production of PHA. By day 61 of operation, the SRT was decreased to 5 days to favor the organisms with higher PHA accumulation capacity. The microbial community characterization revealed that *Betaproteobacteria* class was the dominant one, over *Alphaproteobacteria* and *Gammaproteobacteria*. *Thauera* was the dominant genus during the operation, coming up, however, *Amaricoccus* and *Zooglea* with the change of the operational parameters (Moita and Lemos, 2012).

A few works have been done regarding the valorization of pulp and paper side streams. Using fermented paper mill wastewater, that after fermentation 74% of the soluble COD was present as SCOA (acetic, propionic, butyric and valeric acids). The SBR was operated at a pH-value of 7.3, HRT of 0.2 and 2.2 days and SRT of 7 days. The enriched culture was mainly composed of filaments and floc-forming bacteria, all of them showed PHA production as detected after Sudan Black or Nile Blue staining. The average PHA content was 11% of cdw. After batch accumulation test the maximum PHA content achieved was 48% of cdw, consisted of 31–47 mol-% 3HB and 53–69 mol-% 3HV (Bengtsson et al., 2008b).

By applying a three stage process to select a PHA-storing MMC using paper mill effluent as feedstock an enrichment dominated by *Plasticicumulans acidivorans* resulted, accounting for 56% of the microbial population (Jiang et al., 2012). Activated sludge from a nutrient sewage removal treatment plant was acclimatized, for 5 months, to fermented paper mill wastewater, resulting in the highest amount of PHA so far stored by an MMC fed with real substrate, 77% P(3HB-*co*-3HV) (3HB:3HV = 86:14) in cdw (Jiang et al., 2012).

HSSL is a by-product from the pulp and paper industry with a high COD content, namely lignosulphonates (60 – 80 g L^{-1}), xylose (35 – 45 g L^{-1}) and acetic acid (8 – 9 g L^{-1}). Queirós et al. (2014) studied the possibility of using the residue as substrate to select a PHA-storing enriched culture in a two-step process. An ADF SBR was operated to target the acetic acid consumption. HRT and SRT of 1 and 5 days, respectively, were imposed alongside with 4.2 gCOD L^{-1} day^{-1} of OLR. Authors obtained an P(3HB) accumulation of up to 65% of cdw, and observed that not only the acetic acid was involved in the PHA storage metabolic pathway, but also the xylose and other components. However, a stationary-

state was not reached, revealed by the PHA accumulation fluctuations and the microbial evolution. By FISH, authors were able to identified *Alphaproteobacteria* as the dominant PHA-storing community, accounting for 72.74% after 65 days of operation. Due to the tendency observed, the culture was still adapting to the conditions (Queirós et al., 2014). Later, with a different batch of HSSL, more than 250 days were needed to reach a pseudo-steady state (Queirós et al., 2016).

Biodiesel production originates high amounts of crude glycerol. Such by-product was already explored for PHA production by an MMC. The selection reactor was operated under ADF conditions with 24 h cycles and 2 and 5 days of HRT and SRT, respectively, and pH-value control between 7.2 – 8.2. The culture reached a steady-state after 8.5 SRTs, showing the ability to consume both glycerol and methanol fraction present in the crude and producing PHA around 6% of cdw (Moita et al., 2014). It was observed that the glycerol fraction was the only one contributing for the two biopolymers stored: P(3HB) and glucose biopolymer. In batch tests, the enriched culture could accumulate 47% PHA in cdw at a productivity of 0.27 gX L^{-1} d^{-1}. However, the productivity can be improved if the crude could be completely redirect to PHA formation (Moita et al., 2014).

Campanari et al. (2014) assessed the feasibility of using olive oil mill effluents (OMEs) as a substrate in biodegradable polymer production. The enrichment of the culture was done in a SBR under “feast and famine” conditions with dephenolized and fermented OME. Authors aimed to optimize the OLR (ranging from 2.40 to 8.40. gCOD L^{-1} d^{-1}). At an OLR of 4.70 gCOD L^{-1} d^{-1}, the culture showed the highest values of storage rate and yield, 339.0 ± 48.0 mg COD $gCOD^{-1}$ h^{-1} and 0.56 ± 0.05 gCOD $gCOD^{-1}$, respectively (Campanari et al., 2014). As observed by Albuquerque et al. (2010), the OLR applied to the SBR had influence on the performance of the PHA-accumulating reactor. Authors calculated that the polymer volumetric productivity, combining the SBR and the accumulation reactor, accounted for 1.50 gPHA L^{-1} d^{-1} (Campanari et al., 2014).

2.3.3.3. Other Possibilities

Despite the above presented processes, AN/AE and ADF, being the most common selection methods, a few others were studied to select the best PHA-storing microorganisms or even to eliminate this step.

A 2-stage continuous stirred tank reactor (CSTR) system (under "Feast and Famine" conditions) was used to enrich an MMC in PHA-storing microorganisms using fermented molasses as feedstock. The enrichment step was designed to impose "feast" and "famine" conditions in different reactors, by feeding the carbon substrate to the first reactor (Feast reactor), and the resulting effluent passing by overflow to the second reactor (Famine reactor). The imposed HRT and SRT were 1 and 10 days, respectively. Two parallel systems allowed to study the effect of influent substrate concentration and Feast-to-Famine (F/F) HRT ratio between the reactors. Best results were obtained when high influent concentrations and low F:F HRT ratio were introduced in the reactor, leading to polymer yield on substrate of 0.59 CmolPHA $CmolSCOA^{-1}$ and growth yield of 0.22 CmolX $CmolSCOA^{-1}$. Conversely the Famine growth yield reached higher values 0.42 CmolX $CmolSCOA^{-1}$. In this case a stable PHA content of 23% of cdw was recorded, whereas a content 61% PHA in cdw was attained in the batch test (Albuquerque et al., 2010a).

Recently, an attempt to eliminate the MMC enrichment step was done (Cavaillé et al., 2013). Waste activated sludge (WAS) was induced to accumulate P(3HB) in a fed-batch reactor operated with growth limitation by phosphorus and using acetic acid as substrate. The behavior of WAS from three different sites by applying three defined proportions of carbon and phosphorus to each culture was investigated, resulting in PHA accumulations of 67, 57 and 45% of cdw, respectively (Cavaillé et al., 2013). Through pyrosequencing was possible to infer which bacterial groups were active during P(3HB) accumulation through fed-batch operation from two reactors. Bacteria belonging to the genera *Acinetobacter*, *Zoogloea* and *Simplicispira*, already reported as PHA accumulators, were present in two of the three tests, representing about 40% of total active community. The relative abundance of each genus varied along the operation time. One of the reactors displayed less diversity of active bacteria, recording a strong metabolic

activity from *Methylobacter*. In the other reactor, the culture kept on producing P(3HB) after the first phase (dedicated to the depletion of residual phosphorus) and up to 71 hours of reaction (Cavaillé et al., 2013). This work pointed out that enrichment in PHA-producing microorganisms occurred, observing an increase in organisms already described as PHA accumulators.

Manipulation on the reactor configuration and feeding regimen imposed is also being explored, with an anoxic-aerobic dynamic feeding for enriching an MMC with denitrifying PHA-storing microorganisms. Two parallel bioreactors were set-up: one was operated as conventional activated sludge SBR (AS-SBR) and the second was an integrated biofilm activated sludge (IFAS), a hybrid moving bed-reactor operated in SBR mode (MBBR-SBR) (Anterrieu et al., 2014). The strategy aimed to mimetize the conditions imposed to the water treatment full-scale reactors in a beet sugar factory. Reactors were fed with condensate and wash waters and operated for 24 weeks, with 8 hours cycle and SRT of 16 days and HRT of 4-6 days for AS-SBR and MBBR-SBR, respectively. The highest PHA storage yields were reached at the end of the operations (20 - 24 weeks), 0.77 and 0.79 gCOD $gCOD^{-1}$, for AS- and MBBR-SBRs. Both SBRs presented, thus, biomass with PHA production potential while sustaining process water treatment for carbon, nitrogen and phosphorus for the factory process waters. PHA accumulation reached 60% of cdw for the MBBR-SBR which also presented better nitrification performance. A copolymer P(3HB-*co*-3HV) (3HB:3HV = 85:15 on a molar basis) was produced from the beet tail press water (Anterrieu et al., 2014).

2.4. Applications

Due to the high diversity of monomers and the possibility to combine different ones into the final polymer, a wide range of properties can be obtained for this class of polymers, from thermoplastics to elastomers. Although PHA can replace many types of petrol-derived plastics their production price is not yet commercially competitive. P(3HB) is stiff and brittle, limiting the number of useful applications. However, the utilization of copolymers with other monomers than 3HB, improve their properties. Copolymers of 3HB and 3HV from MMC do not differ from those obtained using pure cultures in terms of

their properties (Laycock et al., 2013; Serafim et al., 2008b). PHA produced by pure cultures have been used in many combinations with other biologically-derived polymers (polylatic acid, dextran, dextrin, amylose, other polysaccharides) or petrochemical-derived polymers (nylon, polyethylene oxide, polyvinyl acetate, polyvinyl chloride) and plasticizers resulting in the modification of other polymers properties in terms of processing and degradability, increasing PHA market share (Philip et al., 2007). These combinations could be also possible for PHA obtained from MMC.

Despite the initial applications of PHA related with everyday consumer goods, particularly in the packaging area due to their good barrier properties, for food packaging and for making plastic beverage bottles (Zhang et al., 2009), other possible applications were tested. They include shampoo bottles, packaging films used for containers and paper coatings, printing and photographic materials, disposable items such as razors, utensils, diapers, feminine hygiene products, cosmetic containers, and cups as well as medical surgical garments, upholstery, carpet, compostable bags and lids, or tubs for thermoformed articles (Chen, 2010). Due to the origin of microorganisms and the substrates used, PHA by MMC should be targeted to less noble applications, where biomedical and pharmaceutical ones are not primary goals.

PHA can be used as a starting material in chemical industry, due to their chiral enantiomeric pure nature, or as raw material for the production of paints. PHA monomers after esterification, using 3-hydroxybutyrate methyl ester (3HBME) and mcl 3-hydroxyalkanoate methyl ester (3HAME) from P(3HB) and PHA_{MCL}, can be used as biofuels (Lopez et al., 2015).

In the agriculture area, PHA are used in the production of devices for controlled release of fertilizers, herbicides and insecticides (Zhang et al., 2009). They are also important growth promoters for plants, using bacterial PHA producers as inoculants, contributing to rhizosphere adaptation, root colonization, and plant growth promotion abilities. Their impact in symbiotic nitrogen-fixing is also acknowledge as well as contributing to a successful competitiveness in the nodulation process in different species of rhizobia (Verlinden et al., 2007). Encapsulation of seeds, biodegradable films for crop

protection or in the construction of greenhouses are other possibilities of utilization in this field (Verlinden et al., 2007).

For bioremediation purposes MMC with PHA accumulation capacity can also be used, namely for oil-contaminated sites using different hydrocarbons such as benzene, toluene, and xylene (BTX) for PHA production, and providing the polymer a competitive advantage to adapt to stresses prevailing in the environment (Nikodinovic et al., 2008). Also, PHA plays an important role in reductive dechlorination (Baric et al., 2012). A natural occurring process where PHA also present an important role is on the treatment of wastewaters for the removal of organic and inorganic contaminants, as phosphorus and nitrogen.

2.5. Short-Chain Organic Acids

SCOA are aliphatic monocarboxylic acids composed by six or fewer carbon atoms (Lee et al., 2014). Due to their low boiling points associated with their low molecular weight, most of these organic acids are considered volatile. The most known and abundant SCOA are acetic, propionic and butyric acids (Zygmunt and Banel, 2009). Also, lactic and valeric acids are considered SCOA.

SCOA are mostly used in food and beverages fields as acidifiers but also in the pharmaceutical and chemical production fields. They are commonly used in food industry as taste enhancing additives and preservatives, in the pharmaceutical industry as buffer solutions, in the cosmetics industry in moisturizers, skin-lightening or anti-acne agents and in the chemical industry for the synthesis of biodegradable polymers or as building blocks for the production of many organic compounds such as alcohols, ketones, esters, among others. Also, SCOA have an important role as intermediates in many biological processes (Singhania et al., 2013; Zacharof and Lovitt, 2013). SCOA can be produced by chemical or biological routes either from fossil resources and renewable resources (Yang, 2007). Acetic acid is considered the most important SCOA commercially, since it covers a great part of the market size globally. The major portion of this acetic acid production is made from petrochemical feedstock through chemical processes, such as acetaldehyde or ethylene oxidation or methanol carbonylation (Zacharof and Lovitt, 2013).

2.5.1. SCOA Biological Production

Pure sugars such as glucose or sucrose have been commonly employed as the main carbon sources for SCOA production. Though, the use of sugars raised economical and ethical problems related to the use of food to produce chemicals during the last years. The use of organic-rich wastes (e.g. food waste, wastewaters, wood waste, among others) to produce SCOA provides a sustainable alternative route, reducing, thus, the constantly increasing amount of waste generated (Lee et al., 2014). Some studies, extensively reviewed by Lee et al. (2014), already reported the use of industrial organic-rich wastes as olive oil mill effluents (Dionisi et al., 2005b), food waste (Jiang et al., 2013; Lim et al., 2008; Yin et al., 2016), waste activated sludge (Jankowska et al., 2015; Ma et al., 2016), pulp and paper mill effluents (Bengtsson et al., 2008a), cheese whey (Bengtsson et al., 2008a; Gouveia et al., 2016; Silva et al., 2013), sugar cane molasses (Duque et al., 2014).

The biologic production of SCOA is based on anaerobic digestion (AnD). AnD is a sequential biochemical process wherein the complex organic compounds present in the waste, such as polysaccharides, lipids and proteins, are hydrolyzed and fermented into intermediate products that are finally converted into methane and carbon dioxide. This process is therefore composed by successive four stages that occur synergistically and in each stage the product of one reaction becomes the substrate for the next reaction. The stages are, by order, hydrolysis, acidogenesis, acetogenesis and methanogenesis (Fig. 2.7). All of the stages are executed by four distinct groups of microorganisms that work in a balanced and sensitive symbiotic relationship (Saady, 2013; Singhania et al., 2013).

During hydrolysis, complex organic polymers such as polysaccharides, are fragmented into their simpler organic monomers by enzymes excreted by hydrolytic fermentative bacteria. Usually, hydrolysis is considered the rate-limiting step of AnD given the difficulty of certain substrates fragmentation. Then, in acidogenesis, also known as acidogenic fermentation (AF), occurs the conversion of these monomers into SCOA, such as acetic, propionic, lactic, butyric and valeric acids, ethanol, carbon dioxide and hydrogen by the same group of microorganisms. These bacteria are facultative anaerobes

(Visvanathan and Abeynayaka, 2012) and those with the highest energetic advantage, since they present the lowest time of replication (≈ 30 minutes) and the highest growth rates of all microorganisms involved in AnD process. Thus, and considering that the substrate is in its monomeric form, the AF stage hardly becomes the limiting stage of the process (Aquino and Chernicharo, 2005; Visvanathan and Abeynayaka, 2012; Zygmunt and Banel, 2009).

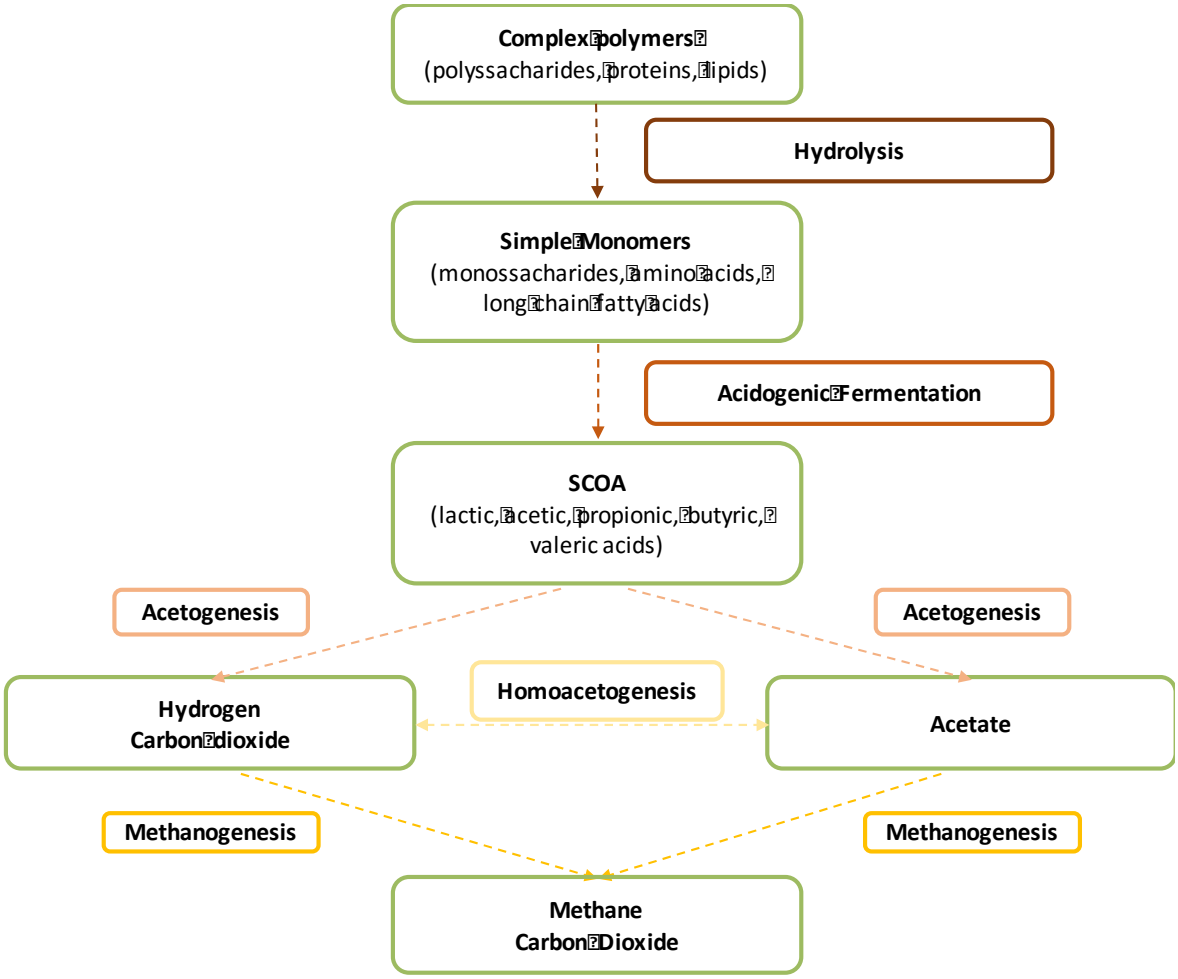

Fig. 2.7. Representation of the AnD process, based on Lee et al. (2014).

Through acetogenesis, SCOA produced in AF are converted by acetogenic bacteria into acetic acid, hydrogen and carbon dioxide. In this stage, homoacetogenic bacteria with the capacity of convert hydrogen and carbon dioxide into acetic acid dominate (Saady, 2013). Lastly, methanogenesis is the conversion of the products obtained in the previous steps into methane and carbon dioxide by methanogenic microorganisms. These

microorganisms can be classified as archaea and restrict anaerobes (Ma et al., 2016; Visvanathan and Abeynayaka, 2012) and subdivided into two groups: acetoclastic methanogenic and hydrogenotrophic microorganisms. The first group, that converts acetic acid into methane, exhibits low growth kinetics (replication time of two to three days) and an extreme sensitivity to environmental changes. The second group also produces methane, although from the conversion of the carbon dioxide. This group presents a faster growth (replication time of at least 6 hours) and contributes for around 30% of the methane achieved at the end of the process (Aquino and Chernicharo, 2005; Jie et al., 2014).

Bearing in mind the sequential process of AnD, isolating the first two step and interrupting the methane production allow to produce SCOA. For that, independent bioreactors should be operated with the imposition of conditions that will select an acidogenic community able to overtake the methanogenic one (Fig 2.8). Also, the SCOA profile produced should be tuned according to the operator objectives through the bioreactor operation.

2.5.1.1. Operational Conditions of Acidogenic Fermentation

The process of AF is strongly affected by the operating conditions such as the origin of the inoculum, temperature, pH, nutrients, organic loading rate (OLR), hydraulic retention time (HRT) and sludge retention time (SRT). Their optimization and control are crucial for the success of acidification. Consequently, it is necessary to establish a strategy that combines the use of the appropriate type of reactor with its optimal operational conditions. Furthermore, it is important to refer that the effect of these conditions in AF depends significantly on each other and on the substrate used (Bengtsson et al., 2008a; Jankowska et al., 2015).

The first important aspect relies on the culture selection for AF. MMC, as already mentioned, can reduce the energy consumption and are able to metabolize complex substrates aerobically (AMMC) or anaerobically (AnMMC) (Wang et al., 2014). In general, microbial cultures used for AF are AnMMC since AnD is an anaerobic process. However, AMMC present great potential to produce SCOA. The fact that aerobic cultures are

subjected to more extreme conditions in aerobic tanks when compared to anaerobic tanks, suggests that AMMC could be more robust than AnMMC and thus, their use as inoculum for AF brings advantages to the operational control of the biological system. Furthermore, as referred before, methanogenic microorganisms are restrict anaerobes and AMMC are composed by aerobes and facultative anaerobes. So, the selection of acidogenic bacteria from AMMC could be more efficient since it could be a way to inhibit the growth of methanogens.

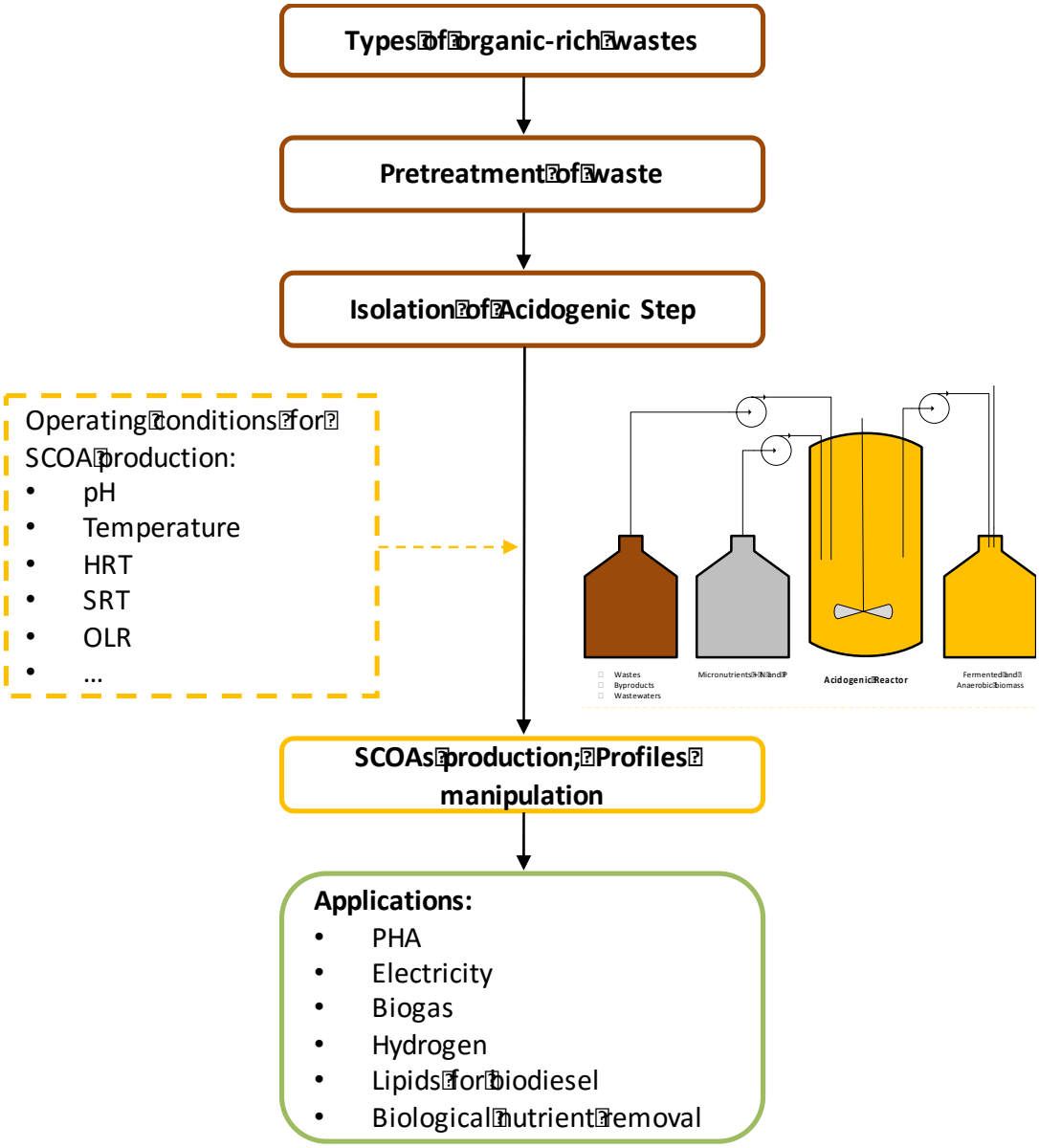

Fig. 2.8. Acidogenic fermentation process and operational parameters used to manipulate the types of SCOA produced.

In general, microorganisms involved in AF tolerate well changes in temperature since they do not surpass the upper limit value in which the decay rate start to exceed the growth rate (Rajeshwari et al., 2000). Nevertheless, operation at a thermophilic temperature requires major costs due to the high-energy input necessary to maintain the high temperatures throughout the process. Thereby, the most appropriate range for this process is mesophilic, since at this temperature range, AF is stable and efficient and does not require great energy input (Jiang et al., 2013). Relatively to the types of SCOA produced, temperature does not affect them significantly, especially in mesophilic range (Lee et al., 2014).

In AF, pH is usually considered the key operational parameter. The values of pH chosen to perform acidification of organic wastes are decisive not only to the success of AF but also influences SCOA profiles obtained in the process. Acidogenic bacteria have a wider range of pH values in which their activity and growth are not affected. However, very extreme acidic (pH 3) or alkaline (pH 12) conditions are known to inhibit acidogenic bacteria (Jie et al., 2014). Hence, the study of the optimal pH value is determinant for each substrate used (Tamis et al., 2015). As an example, Jie et al. (2014) showed that to produce SCOA from excess sludge the optimum pH value was 10.0. In general, for this type of substrate, an alkaline value of pH in a range of 8 – 11 is desirable, not only because at these values the activity of methanogens suffers inhibition but also due to the fact that alkaline conditions promote the hydrolysis of sludge, thus increasing the availability of the soluble substrate to conversion (Jie et al., 2014; Lee et al., 2014). On the contrary, Jiang et al. (2013) demonstrated that a neutral pH value of 6 was the optimum to produce SCOA from food waste. Also, they showed that extreme acidic values of pH, near to 3, led to a low SCOA concentration, which could be explained by the fact that, at this value of pH, SCOA are undissociated. Thus, microbial growth suffers inhibition since more energy is required for maintaining intracellular pH by actively pumping out undissociated SCOA that diffuse over the cell membrane into the cell (Jiang et al., 2013; Tamis et al., 2015). Relatively to the production of SCOA from cheese whey, more acidic pH values are required to a better performance of conversion, with an optimum between

5.25 and 5.5, while for paper mill effluents, an optimum range of 5.5 – 6 is required (Bengtsson et al., 2008a).

The type of SCOA produced are also influenced by pH values chosen. For instance, for the AF of dairy wastewater, the production of propionic acid was found to be enhanced at pH 4 - 4.5, whereas the production of acetic and butyric acids was favored at pH 6 – 6.5 (Yu and Fang, 2002). On the other hand, for cheese whey, the opposite was observed since the propionic acid production increased with pH increasing from 5.25 to 6 while the acetic and butyric production decreased. In the case of pulp mill effluent, butyric and propionic acids increased with pH values in the range of 4.9 – 6, whereas acetic acid decreased (Bengtsson et al., 2008a).

Retention times of substrate and microbial population are operational parameters that greatly affect the success of acidification. In general, longer HRT values are recommended for AF processes, since they allow for more time for the culture to adapt and convert the substrate efficiently. This is important because most of the substrates used for AF are recalcitrant, not easily biodegradable and consequently, it is difficult for the MMC to quickly adapt and convert them. On the other hand, shorter HRT can lead to the washout of biomass. The operation at higher HRT represents a major problem since it requires large reactors, thus increasing significantly the cost of the process (Lee et al., 2014). From another point of view, shorter HRT prevent the growth of methanogenic microorganisms, since they have low growth rates compared to acidogens (Jankowska et al., 2015). Consequently, the choice of the most appropriate HRT must take into account these factors to achieve a satisfying yield with the lowest cost possible.

2.6. Substrate and Lignocellulosic Biorefinery Concept

The substrate cost is the one of the main contributors to the high cost of PHA production. It was estimated that 40 – 50% of the total PHA production cost is due to the type of substrate used (Dias et al., 2006). For this reason, a more cost-effective process should include the selection and preparation of cheap substrates, which can be successfully used by microorganisms to synthetize PHA at high productivities. Also, the substrate must be converted into a polymer with the suitable properties that fit in a wide

range of industrial applications. As mentioned in section 2.3, in the past years, a wide variety of low-cost carbon substrates was used to produce PHA by MMC.

The choice of the substrate should, wherever possible, meet the concept of the biorefinery. Substrates like wastes from several industries (food, agricultural, forest, paper) must integrate production plants that comprise suitable technologies for their conversion into valuable intermediate and final products, fuels and energy (Koutinas et al., 2014). Among the different types of biorefineries, a lignocellulosic-based type could be the most successful because of the abundance and affordability of raw materials, wide variety and good marketing of the bio-based products. Following this, the substrate chosen to be used in this project was the HSSL, originated from the pulping of *Eucalyptus globulus* wood during the pulp production process, Fig. 2.9.

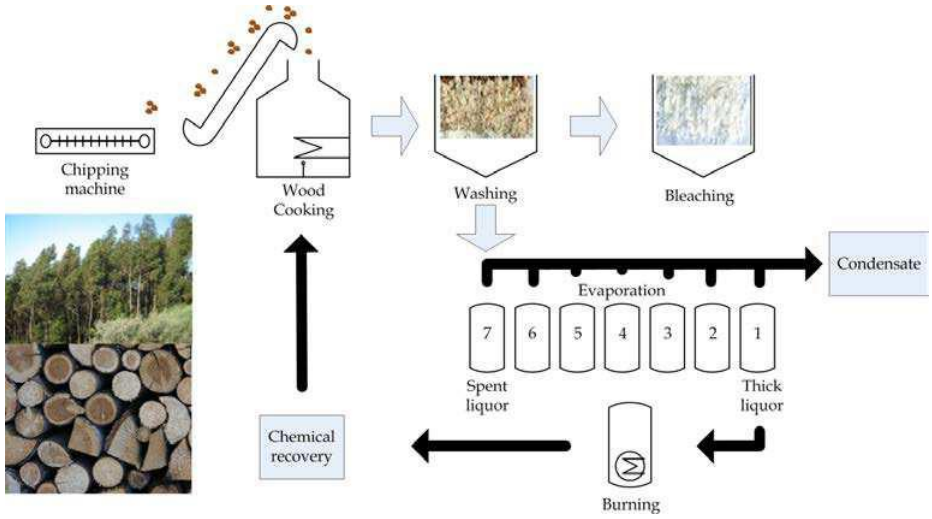

Fig. 2.9. Pulp process using sulfite acid and Mg^{2+} base. The SSL used in this work is a waste product from the process and is collected from the 7^{th} evaporator (from Fernandes et al. (2012)).

This process is carried out in batch digesters under acidic conditions (pH 1 – 2) at 135 – 145 °C for 6 – 12 h (Fernandes et al., 2012). Caima - Indústria de Celulose S.A. (Constância, Portugal) is a production unit from ALTRI group than applies this process. Since June 2015, Caima Mill produces dissolving pulp, being the current technical capacity slightly above 100,000 tonnes/year. Due to the chemical properties of the acid cocking process at Caima (sulphite) it has been designed and implemented a conversion project from BEKP to Dissolving Pulp. Presently, almost all the pulp produced is being sold to

clients in China, that are textile producers. Nevertheless, it is a goal for Caima Mill to continue its systematic development project aiming to reach higher added value market niches such as ethers and/or acetates.

During sulfite pulping process, lignin and part of hemicelluloses (about 50% based on wood) are dissolved in sulfite spent liquors (SSLs) composed by monomeric sugars already in the fermentable form. Roughly one tonne of solid waste is dissolved in the spent liquor (SSL 11 – 14% solids) per ton of pulp produced. SSLs are produced in large amounts, about 90 billion litres annually worldwide (Fernandes et al., 2012). Approximately 8 – 10 m^3 HSSL are produced per tonne of pulp, meaning that Caima Mill, alone, produces around 8 –10 x 10^6 m^3 of HSSL each year.

During the sulfite pulping, lignin is sulphonated and removed from wood as salts of lignosulphonates (LS). A significant part of hemicelluloses is hydrolyzed and removed from wood to spent sulfite liquor. Cellulose is maintained almost intact during acidic sulfite pulping. After the pulping process, unbleached pulp is washed and HSSL containing dissolved LS and degraded carbohydrates are concentrated by evaporation. Concentrated HSSL may be burned for the energy and reagents recovery (case of Na^+ and Mg^{2+} bases) or commercialized (Pereira et al, 2012). The composition of sugars in HSSL depends on the composition of wood processed. A direct correlation between the chemical composition of wood used in pulping and the chemical composition of HSSL is possible when single species are involved (e.g. *Eucalyptus globulus*). In general, the major components of HSSLs are LS followed by the monomers from hydrolyzed hemicelluloses. Pentoses are the dominant sugars in HSSLs, namely xylose (16 – 43 g L^{-1}) and arabinose (1.0 – 21.0 g L^{-1}). Hexoses are present at lower concentrations namely glucose (2.3 – 9.0 g L^{-1}) mannose (1.0 – 9.0 g L^{-1}) and galactose (1.6 – 5.0 g L^{-1}) (Pereira et al, 2012). Among the volatile compounds, acetic acid is the most abundant although furfural is also present but at low concentrations. The significant amounts of acetic acid and xylose in HSSL are the result of extensive degradation of acetylated glucuronoxylan, which is the predominant hemicellulose in hardwoods (Pereira et al, 2012).

Other HSSL components are produced and known to be fermentation inhibitors. They are, conventionally, classified into four different groups according to their origin:

sugar degradation products, lignin degradation products, compounds derived from extractives and heavy metal ions (Fernandes et al., 2012; Pereira et al, 2012). This classification does not include free SO_2 in HSSL, because it is normally eliminated by vapour distillation or by liquor heating under vacuum. Sugar degradation products are formed after polysaccharide hydrolysis by dehydration. Pentoses produce furfural and hexoses are converted in hidroxymethylfurfural (HMF) and levulinic acid. Lignin derivatives constitute another group of microbial inhibitors. In particular, it was found that LS from sulfite pulping of *E. globulus* correspond to partially sulphonated lignin oligomers including monomeric phenolic compounds with one or two the sulphonic groups. Another group of inhibitors includes extractives (polyphenolics, fatty acids and diterpenoids). In general, the extractives are considered less toxic to microbial growth than lignin derivatives. However, some hardwoods (e.g. *E. globulus*) contain high amount of hydrolysable tannins giving rise to gallic and elagic acid and pyrogallol during the pulping. These phenolic compounds are known inhibitors of microbial growth, possessing strong anti-fungal properties (Fernandes et al., 2012; Pereira et al, 2012).

Using HSSL as raw material to produce added value products fits well within the biorefinery concept, to decrease the dependence from fossil resources and to improve the economic sustainability of pulp mills (Xavier et al., 2010). An advantage of HSSL, comparing to agro-forestry residues, lies on lignocellulosic fraction that has already suffered a hydrolysis step releasing most of the monomeric sugars, xylose, mannose and arabinose. However, due to its high toxicity, it is a challenge to valorize the HSSL. Using eco-engineering and an appropriate strategy such fact could be overcome.

Studies performed under ADF conditions showed that the microorganisms from MMC consume, preferentially, the SCOA, like acetic acid (Lemos et al., 2006). For this reason, HSSL is a good candidate as a renewable, inexpensive and sustainable substrate for PHA production by MMC, since it contains a significant amount of acetic acid. If the selected MMC does not consume the sugars, they can be used for a subsequent production of second-generation bioethanol, as proposed by Xavier et al. (2010).

2.7. Conclusion

Although being challenging, PHA production by MMC is also a way of waste treatment through its valorization. Being PHA production a competitive advantage for microbial cultures, the enrichment in PHA-storing bacteria depends more on the operational conditions impose to the process rather than the existence of sterility. PHA producing processes using MMC and waste as substrates could help to reduce the polymer costs but at the same time using the existing facilities of waste/wastewater treatment systems and residues on location could contribute to the overall profit of industries and WWTPs. Queirós et al. (2014) proved possible to produced PHA from HSSL and resorting to an MMC. This is the entrance door to explore the process parameters and configuration to optimize the process valorizing HSSL as carbon source, where SCOA will play a part. Moreover, optimizing the SCOA production is a crucial step not only to be used as substrate and improve the PHA volumetric productivities but also to further valorize the waste streams.

2.8. References

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Chapter 3

PHA-accumulating bacteria isolated from activated sludge acclimatized to HSSL

This work aimed the isolation and characterization of organisms able to store PHA, from MMC selected under feast and famine conditions, using HSSL as carbon source.

The MMC could accumulate PHA from the different HSSL carbon components (acetic acid, xylose and lignosulphonates) with a PHA storage content, during the reactor operation, of 54.2 ± 10.6% cdw P(3HB). To clarify the MMC composition, isolation was performed from the MMC, using HSSL or its main components. Three isolates were identified as *Rhodococcus* sp., *Pseudomonas* sp. and *Klebsiella* sp.. All isolates could grow and accumulate PHA in harsh conditions of HSSL using xylose, the second most abundant sugar in nature. *Rhodococcus* and *Pseudomonas* isolates could accumulate a homopolymer P(3HB), whereas *Klebsiella* accumulated the copolymer P(3HB-*co*-3HV), with a composition of 96% 3HB and 4% 3HV. 16S rDNA clonal analysis was performed to identify the unculturable fraction of the MMC several genera were found to be related to PHA production. The capability of the isolated bacteria to simultaneously consume xylose and accumulate PHA offers a possibility for further studies and routes for the valorization of xylose-rich byproducts.

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3.1. Introduction

PHA are polymers produced biologically, with similar properties to synthetic plastics. PHA are biodegradable, biocompatible and may be produced from renewable sources, thus offering a solution to the environmental hazards of conventional plastics (Gumel et al., 2013). In the last few years, the interest in PHA production by MMC, such as activated sludge from WWTPs, is growing since their use does not require sterile conditions, thus saving energy and equipment costs (Serafim et al., 2008; Villano et al., 2010). In this way, overall production costs can be further decreased. The selection of substrate is an important factor to optimize PHA production since it affects the final cell content, composition and properties of the polymer. Moreover, raw materials can account for 40% of the total operating costs of PHA production, and more than 70% of these costs are related with the carbon source (Reis et al., 2011). Consequently, the use of cheap carbon substrates is attracting a considerable interest (Akaraonye et al., 2010; Serafim et al., 2008). Recently, Queirós et al. (2014) successfully selected an MMC, which under feast and famine conditions could store PHA from HSSL, a by-product of the pulp and paper industry.

Despite the growing importance of PHA production by MMC, numerous researchers have been attempting to isolate PHA-producing microorganisms from different sources aiming to discover and identify robust and novel species with high storage capacity (Sangkharak and Prasertsan, 2012). The isolation of PHA-accumulating bacteria from activated sludge has been the object of several studies (Kourmentza et al., 2009; Liu et al., 2000; Reddy and Mohan, 2012; Wong et al., 2002). The search for strains able to resist and metabolize inhibitory compounds present in sugarcane bagasse hydrolysates was performed by Lopes et al. (2014). *Burkholderia* sp. F24 reached values of biomass of 25 g L^{-1}, with a content of 49% P(3HB) in 44 h, corresponding to a volumetric productivity of 0.28 g L^{-1} h^{-1} (Lopes et al., 2014).

Therefore, the present paper aims to study the microbial population selected by Queirós et al. (2014) to improve the use of HSSL as a substrate in the biorefinery concept. The isolation and characterization of organisms from the MMC was performed to obtain PHA-accumulating bacteria able to grow under harsh conditions brought by complex

substrates, such as HSSL, and to use xylose as carbon source. Additionally, a 16S rRNA gene clonal analysis was carried out to identify the uncultivable PHA-accumulating bacteria present in the MMC.

3.2. Materials and Methods

3.2.1. Culture

Microorganisms isolated in this work were obtained from a PHA-storing MMC selected in a SBR under ADF with HSSL as substrate. The operation of SBR was described by Queirós et al. (2014), however it was not fully optimized, showing evidence of a more stable behavior by the end of the operation. Briefly, the SBR was inoculated with activated sludge obtained from the aerobic tank of the municipal WWTP Aveiro Norte (SIMRia). The SBR, with a working volume of 1.5 L, was operated under ADF conditions, during which alternating feast and famine phases were imposed. The SBR worked in cycles of 12 h that comprised 10.5 h of aerobiosis, with fresh medium supplied during the first 15 min, 1 h of settling (with agitation and aeration switched off) and 0.5 h of withdrawing. The HRT and SRT were 1 and 5 days, respectively.

3.2.2. Culture Medium

HSSL was supplied by Caima - Indústria de Celulose S.A. (Constância, Portugal) and was obtained from a magnesium based acidic sulfite pulping of *Eucalyptus globulus* and pre-treated as previously described (Queirós et al., 2014; Xavier et al., 2010). The HSSL composition was fully characterized by (Marques et al., 2009).

Two different culture media were used to isolate the different microorganisms responsible for the uptake of different substrates. The first culture medium had the same composition of the one fed to the selected MMC in the SBR: pre-treated HSSL and mineral solution (Queirós et al., 2014). The second one consisted of synthetic mineral, salts and vitamins medium (MSV), prepared with the major carbon sources (sodium acetate (CH_3COONa) - 0.172 g L^{-1} and xylose ($C_5H_{10}O_5$) - 0.360 g L^{-1}) present in HSSL (Table 3.1). The MSV medium was also composed by (per liter of MilliQ water): 85 mg KH_2PO_4, 110 mg K_2HPO_4, 100 mg $MgSO_4 \cdot 7H_2O$, 80 mg $CaCl_2 \cdot 2H_2O$, 2 mg $FeCl_3 \cdot H_2O$, 3 mg Na·EDTA and

500 mg $(NH_4)_2SO_4$. 1 mL L^{-1} of vitamin solution, composed by (per liter of MilliQ water): 20 mg Biotin, 20 mg Folic Acid, 100 mg Pyridoxine, 50 mg Thiamine, 50 mg Riboflavin, 50 mg Nicotinic Acid, 50 mg Pantothenic Acid, 2 mg Vitamin B_{12}, 50 mg 4-Aminobenzoic Acid, was added to the growth medium. Agar was used to a concentration of 15 g L^{-1}.

3.2.3. Bacterial Isolation

To isolate PHA-accumulating bacteria from the selected MMC, solid culture medium was prepared, for each media described above. Despite SBR being operated without temperature control, the temperature remained around 20 °C. Therefore, a volume of 50 µL of MMC were spread onto agar growth medium and incubated at 20 °C for 48 h. Pure cultures of PHA-storing bacteria were isolated after repeated streaking of isolated colonies. The PHA-accumulating capacity of the isolates was evaluated through their inoculation on 50 mL Erlenmeyer flasks with culture medium of 0.5 g L^{-1} of acetic acid or xylose, at 20 °C for 48 h followed by Nile blue staining of samples.

Table 3.1. Composition of eucalypt sulfite spent liquor (Xavier et al*.,* 2010).

Components	Concentration (g L^{-1})
Lignosulphonates	78.2 ± 0.6
Acetic Acid	8.2 ± 0.3
Furfural	< 0.1
Ash	19.8 ± 0.2
D-Xylose	24.6 ± 0.5
D-Mannose	8.5 ± 0.9
L-Arabinose	7.8 ± 0.3
D-Galactose	4.5 ± 0.1
D-Glucose	2.3 ± 0.1
L-Rhamnose	1.6 ± 0.3
L-Fucose	0.4 ± 0.3

3.2.4. Kinetic tests

A 400 mL Erlenmeyer flask was inoculated with 50 mL of actively growing cells on culture medium plus the carbon source with the same concentration as in SBR feeding: 0.172 g L^{-1} of sodium acetate or 0.360 g L^{-1} of xylose. Kinetic tests were performed at 20

°C, 90 rpm for 24 h, in triplicate. Samples were taken during tests to evaluate the culture growth and substrate consumption through chemical oxygen demand (COD) analysis. Nile blue staining procedure was performed to evaluate PHA accumulation.

3.2.5. Microscopic Visualizations

Gram-staining was performed according to the procedure described by Jenkins et al., 2003. Nile blue staining was applied to fresh samples collected as described by Ostle and Holt, 1982, with the goal of monitoring the PHA accumulating capacity of the isolates during the incubations. FISH was performed on paraformaldehyde-fixed biomass samples (Amann et al., 1995). Several oligonucleotide probes were applied. All the hybridizations with group-specific probes were carried out simultaneously with probes EUB338, EUB338-II and EUB338-III combined in a mixture (EUB338mix) for the detection of most bacteria, and with DAPI staining for quantifying the total number of cells. All the probes were synthesized with FITC and Cy3 labels and purchased from MWG Biotech (Germany). Samples were visualized using an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software).

3.2.6. Analytical Methods

Overall substrate consumption was determined based on COD evolution, using Spectroquant® photometric kit (Merck), according to the manufacturer's instructions. The amount of biomass was accounted as cell dry weight. A sample of 50 mL was filtered using previously dried and weighed membranes (cellulose acetate filter, 0.2 µm pore size). Then, the membranes were placed in an oven at 105 °C for 72 h. After cooling and weighing, the biomass concentration was determined in g L^{-1} of suspended solids (SS). PHA concentration was determined by gas chromatography following the method described by Lemos et al. (2006).

3.2.7. Genomic DNA extraction and PCR amplification of 16S rRNA gene

DNA was extracted from 2 mL of the MMC sample following the protocol reported in Rossetti et al., 2003. The concentration and purity of the genomic DNA were

determined by NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). 16S rRNA genes of the isolates and genomic DNA were amplified using primers 27 F and 1492R for the Bacteria domain using the TaKaRa Ex TaqTM kit (Japan) as previously described (Rossetti et al., 2003). T7f and U19r (or M13r), which are specific plasmid primers, were used for the screening of clones from the 16S rDNA clone library (Table 3.2). PCR products were purified using the QIAquick® PCR purification kit (Qiagen, Milan, Italy). 16S rRNA gene sequences of the clone inserts were obtained using the following primers: 530f, 926f, 907r and 519r (Table 3.2).

3.2.8. Cloning of 16S rRNA gene

Cloning of PCR products was carried out using pGEM-T Easy Vector System (Promega, USA) into *Escherichia coli* JM109 competent cells (Promega) according to the manufacturer's instructions. Positive inserts were amplified from recombinant plasmids obtained from white colonies by PCR using the sequencing primers T7f and M13r following the PCR protocol previously described.

Table 3.2. Primers used in PCR and sequencing.

	Primers	Sequence (5' – 3')
PCR	27f	AGAGTTTGATCMTGGCTCAG
	1492r	TACGGYTACCTTGTTACGACTT
	T7f	TAATACGACTCACTATAGGG
	U19r	GTTTTCCCAGTCACGACGT
	M13r	TCACACAGGAAACAGCTATGAC
Sequencing	530f	GTGCCAGCMGCCGCCG
	926f	AAACTYAAAKGAATTGACGG
	907r	CCGTCAATTCMTTTRAGTTT
	519r	GWATTACCGCGGCKGCTG

M = C:A; Y = C:T; K = G:T; R = A:G; W = A:T; all 1:1

The nucleotide sequences identified in this study were deposited in the GenBank database under accession numbers KM873626, KM873627 and KM873628, for the isolates AF1, AF2 and DS1, respectively. For the clones FA1, FA2, FA3, FA4, FA5 and FA6,

the accession numbers were KM873629, KM873630, KM873631, KM873632, KM873633 and KM873634, respectively.

3.3. Results and Discussion

3.3.1. SBR Operation

The MMC used in this study was selected by operating a SBR under ADF conditions with HSSL as substrate, with an OLR of 4.2 gCOD L^{-1} d^{-1}, during 67 days without any interruptions. SBR was inoculated with activated sludge obtained from the aerobic tank of the municipal wastewater treatment plant, Aveiro Norte (SIMRia). The SBR worked in cycles of 12 h, with a HRT of 1 day and SRT of 5 days (Queirós et al., 2014). Queirós et al. (2014) described the SBR operation in more detail and the evolution of the microbial community. They observed that *Alphaproteobacteria* was clearly the dominant class (Fig. 3.1) at the end of the reactor operation, when the SBR reached a pseudo-steady state. This observation was based on the stabilization of polymer accumulation in the last 20 days of operation, 53.25 ± 3.25% PHA. For the same period, a stable microbial population was observed with minor fluctuations of *Alphaproteobacteria*, 75.33 ± 2.44% of the total bacteria. To a minor extent, cells belonging to *Betaproteobacteria* and *Gammaproteobacteria* were also detected by FISH analysis. The MMC showed a quite high PHA storage capacity with an average value of storage content of 54.2 ± 10.6% P(3HB) (Queirós et al., 2014). Hence, the isolation of microorganisms able to consume xylose, accumulate PHA and survive in such harsh conditions was important to understand the community dynamics and establish a starting point to a possible process of PHA production. As xylose is the second most abundant sugar in nature, it is important to indicate microorganisms able to convert it into other value added products. Moreover, with the increasing research in using lignocellulosic biomass as substrate for several biological processes, those microorganisms are gaining importance.

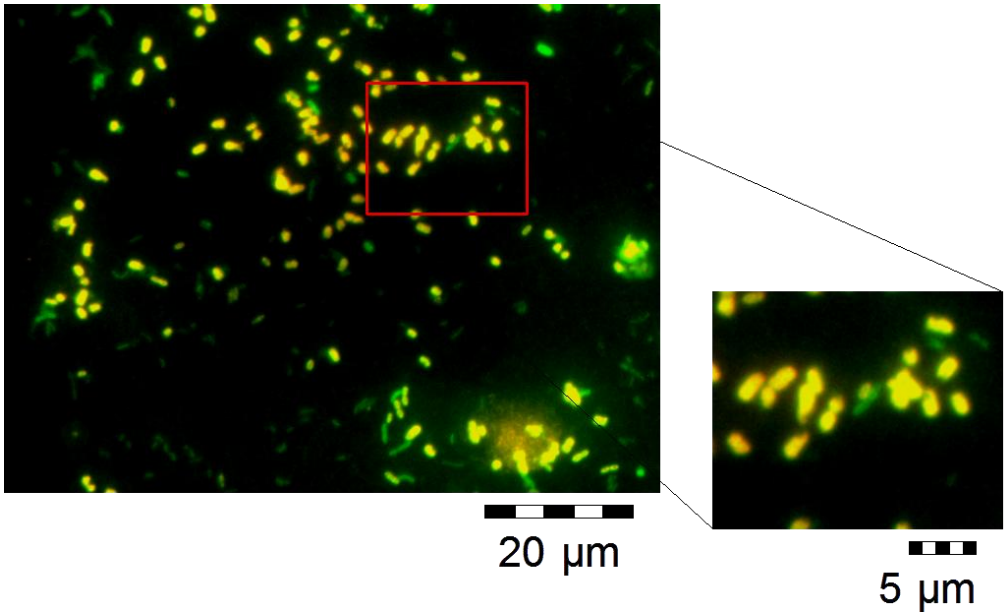

Fig. 3.1. FISH picture from SBR last day of operation. Cells in yellow were hybridize with *Alphaproteobacteria* specific probe and green cells hybridize with EUB338mix probe.

During the SBR cycles, the concentrations of main carbon sources of HSSL decreased along with ammonium. The MMC revealed a preference towards acetic acid, being completely consumed after 4 h of reaction at a rate of 0.110 g L^{-1} h^{-1}. The ammonium uptake rate was faster before the acetic acid exhaustion, 0.177 g L^{-1} h^{-1} decreasing to 0.074 g L^{-1} h^{-1}. Xylose was consumed along with acetic acid but at a slower rate, around 0.030 g L^{-1} h^{-1}, but never depleted, remaining around 70% at the end of each cycle. Concerning the lignosulfonates, there was not a clear tendency in any of the cycles, but it was consumed at a rate of 0.098 g L^{-1} h^{-1} (Queirós et al., 2014). Fig. 3.2. shows an example of an SBR cycle, in which the accumulation reached 19.5%, with an $Y_{PHA/S}$ of 0.44 gPHA gS^{-1}. Considering only acetic acid as the main precursor for PHA production, the $Y_{PHA/Acet}$ increased to 1.04, suggesting that this substrate was not the only one involved in PHA accumulation. This was confirmed by Queirós et al. (2014) during batch tests with acetic acid and xylose fed separately. In these tests, the MCC was able to accumulate PHA from both carbon sources, reaching storage contents of 63.4 and 31.5%, respectively (Queirós et al., 2014).

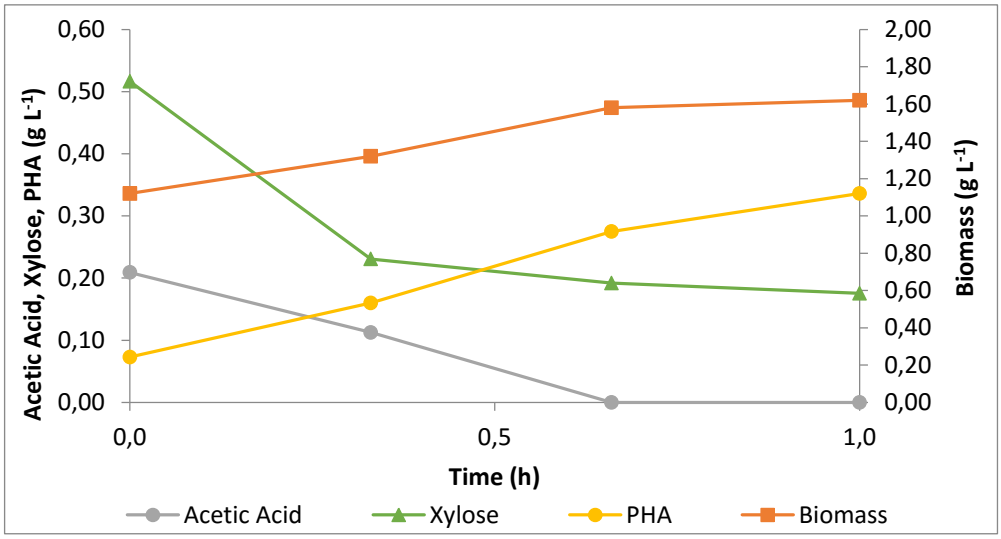

Fig. 3.2. SBR cycle example. In each cycle the acetic acid, xylose, PHA and biomass evolution were followed.

3.3.2. Characterization of the isolates and PHA storage

The isolation attempts of PHA-accumulating bacteria able to survive in harsh conditions were first performed on solid medium containing the same composition of that supplied to the SBR. From two different Petri dishes inoculated with the MMC, five isolated colonies from around 150 grown were selected randomly, continuously streaked onto solid medium and named AF1 to AF5. Additionally, synthetic mineral medium, MSV, containing sodium acetate or xylose, the main carbon components of HSSL, was tested. Colonies only grew with xylose as the sole carbon source. After several replating cycles, a pure culture was obtained, and called DS1.

Partial sequencing of 16S rRNA genes of AF1 and AF3 isolates showed 100% similarity with *Rhodococcus* sp. while isolates AF2, AF4 and AF5 had 100% similarity with *Pseudomonas* sp. The complete sequencing of each representative of isolates belonging to *Rhodococcus* sp. (AF1) and to *Pseudomonas* sp. (AF2) was performed, and hereafter experiments were performed only using AF1 and AF2 isolates. Near full length 16S rRNA gene sequence of AF1 showed that this isolate was closely related to *Rhodococcus qingshengii* strain djl-6, with 100% of sequence similarity (Xu et al., 2007). The AF2 isolate had 99% sequence similarity to *Pseudomonas libanensis* strain CIP 105460 (Dabboussi et al., 1999). The complete sequencing of 16S rRNA genes of DS1 isolate allowed for its

identification as *Klebsiella* spp., sharing 99% similarity with an uncultured *Klebsiella* sp. clone JXS1-28 (Peng et al., 2012).

The PHA accumulating ability of the isolates was evaluated after their inoculation on mineral base medium with sodium acetate at a concentration of 0.5 g L^{-1} followed by Nile blue staining of samples. This specific staining for PHA inclusions confirmed that the isolates were PHA accumulating bacteria. The presence of inclusion bodies of PHA was easily observed by the presence of bright dots in the cells after the Nile blue staining procedure under epifluorescence microscopy (Fig. 3.3).

Kinetic tests were performed to characterize isolates AF1, AF2 and DS1 and evaluate growth on different carbon sources and PHA-accumulating behavior, namely on HSSL and xylose, the main component of HSSL and a carbon source with limited use among microorganisms (Lopes et al., 2009). The results of the kinetic parameters obtained are summarized in Table 3.3. Growth and substrate consumption in the three-different media, for AF1 and AF2, are shown in Figs. 3.4 and 3.5, respectively. Diversely from isolates AF1 and AF2, acetate did not sustain the growth of DS1, which only grew on xylose as sole carbon source (Fig. 3.6). Members of *Klebsiella* were described as able to grow on xylose and to store PHA and are often found in activated sludge systems (Lopes et al., 2009).

Table 3.3. Main parameters obtained from the kinetic batch tests of isolates AF1, AF2 and DS1.

Isolate	Carbon Source	μ_{max} (h^{-1})	q_s ($gS\ gX^{-1}\ h^{-1}$)	$Y_{(X/S)}$ ($gX\ gS^{-1}$)
AF1	HSSL	0.212 ± 0.0219	0.466	0.454
	Acetic Acid	0.153 ± 0.0252	0.519	0.295
	Xylose	0.188 ± 0.0238	0.572	0.329
AF2	HSSL	0.251 ± 0.0526	0.336	0.747
	Acetic Acid	0.194 ± 0.0147	0.430	0.451
	Xylose	0.130 ± 0.00578	0.264	0.492
DS1	Xylose	0.316	1.66	0.191

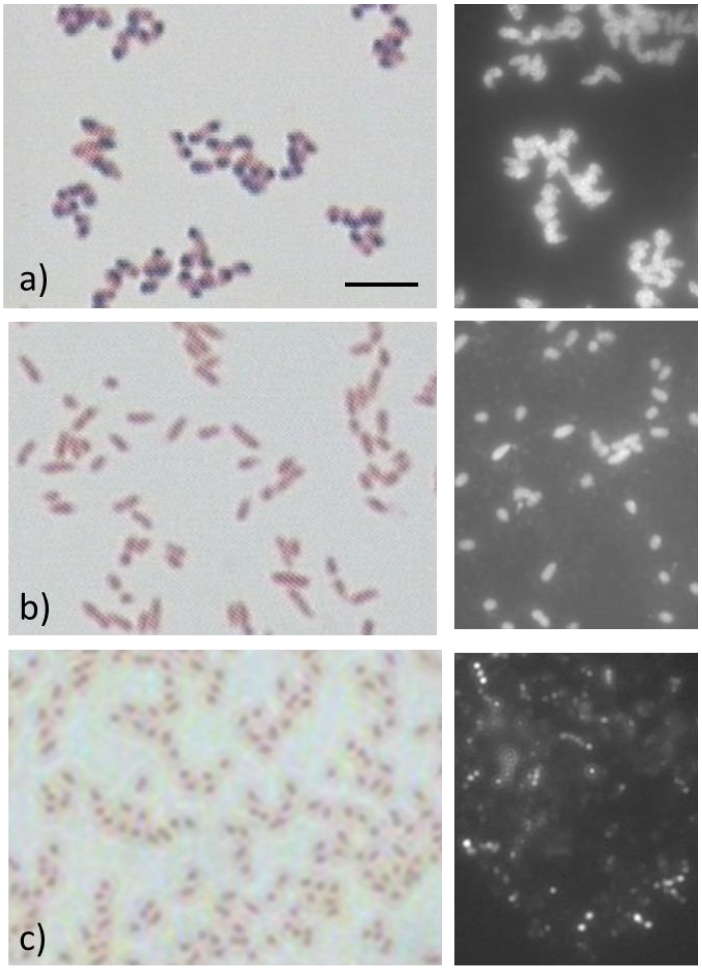

Fig. 3.3. Gram and Nile Blue staining. Appearance of the isolates a) AF1, b) AF2 and c) DS1 after Gram (left side) and Nile Blue staining showing intracellular PHA storage (right side). Bar is 5 µm.

Isolate AF1 showed a preference for medium containing HSSL or xylose, since it began to grow right after inoculation with an undetectable lag phase in both media. With acetic acid, as sole carbon source, the isolate presented a lag phase of around 2 h. A higher biomass concentration, of 0.510 ± 0.0966 gVSS L^{-1}, was obtained in the assay with HSSL (Fig. 3.4). This value was 6 times higher than the biomass concentration obtained using xylose as sole carbon source, 0.0867 ± 0.0538 gVSS L^{-1}, and 17 times higher than using acetic acid, 0.0308 ± 0.00247 gVSS L^{-1}. One reason for the differences observed in biomass amount may be the lower concentration of substrate in media with acetic acid and xylose, since the amount of carbon sources were determined to be approximately the same as in HSSL but other carbon compounds are missing; sugars like mannose, galactose, arabinose and phenolic components such as pyrogallol and galic acids that

could be metabolized by the organisms. However, xylose and acetic acid were still present at the end of the kinetic tests, with acetic acid at much lower concentration (0.0467 gCOD L^{-1}) than xylose (0.124 gCOD L^{-1}), with cells already in the death phase, showing that other nutrients besides carbon were missing. The lack of other components present in HSSL needed for microbial growth could be supported by the value of specific growth rate of isolate AF1 when grown in liquid medium with HSSL, 0.212 ± 0.0219 h^{-1}, which was the highest showed by this isolate. With acetic acid, as sole carbon source, AF1 presented a maximum growth rate (μ_{max}) of 0.153 ± 0.0252 h^{-1} and in xylose a μ_{max} of 0.188 ± 0.0238 h^{-1}. Growth and PHA accumulation by *Rhodococcus* strains using acetate as carbon source has already been reported, but the authors only quantified PHA accumulation content and monomeric composition, without information about growth (Haywood et al., 1991; Hori et al., 2009). However, the effect of acetate concentration on cell growth of a *Rhodococcus* strain, *R. rhodochrous*, has been previously discussed (Honda et al., 1998). According to Honda et al. (1998), cell growth was significantly inhibited at concentrations of acetate above 3 g L^{-1}. Below 2.5 g L^{-1}, the growth rate was in a range of 0.15 to 0.16 h^{-1}, which confirmed the results obtained with isolate AF1. The fact that AF1 could metabolize xylose was a very interesting finding, since no reports on utilization of this sugar by wildtype strains belonging to genus *Rhodococcus* have thus far been found.

Isolate AF2 identified as *Pseudomonas* sp. showed a similar behavior to AF1, since it also grew in the three media, obtaining higher biomass concentrations when grown in HSSL. However, the biomass concentrations obtained were lower than those obtained with AF1. In HSSL, the biomass concentration obtained for AF2, 0.174 ± 0.0297 gVSS L^{-1}, was three times lower than for AF1 in the same medium. The biomass concentration achieved in HSSL was about three times higher than in the assays using acetic acid or xylose as sole carbon sources, which were 0.0536 ± 0.0121 gVSS L^{-1} and 0.0642 ± 0.0124 gVSS L^{-1}, respectively. Table 3.3 shows the preference of both isolates for growing in medium with HSSL: a higher μ_{max} in the essays with HSSL, 0.212 ± 0.0219 h^{-1} and 0.251 ± 0.0526 h^{-1}, and yield biomass on substrate ($Y_{X/S}$), 0.454 gX gS^{-1} and 0.747 gX gS^{-1}, were obtained by AF1 and AF2, respectively. On the other hand, AF1 (*Rhodococcus* spp.) shown

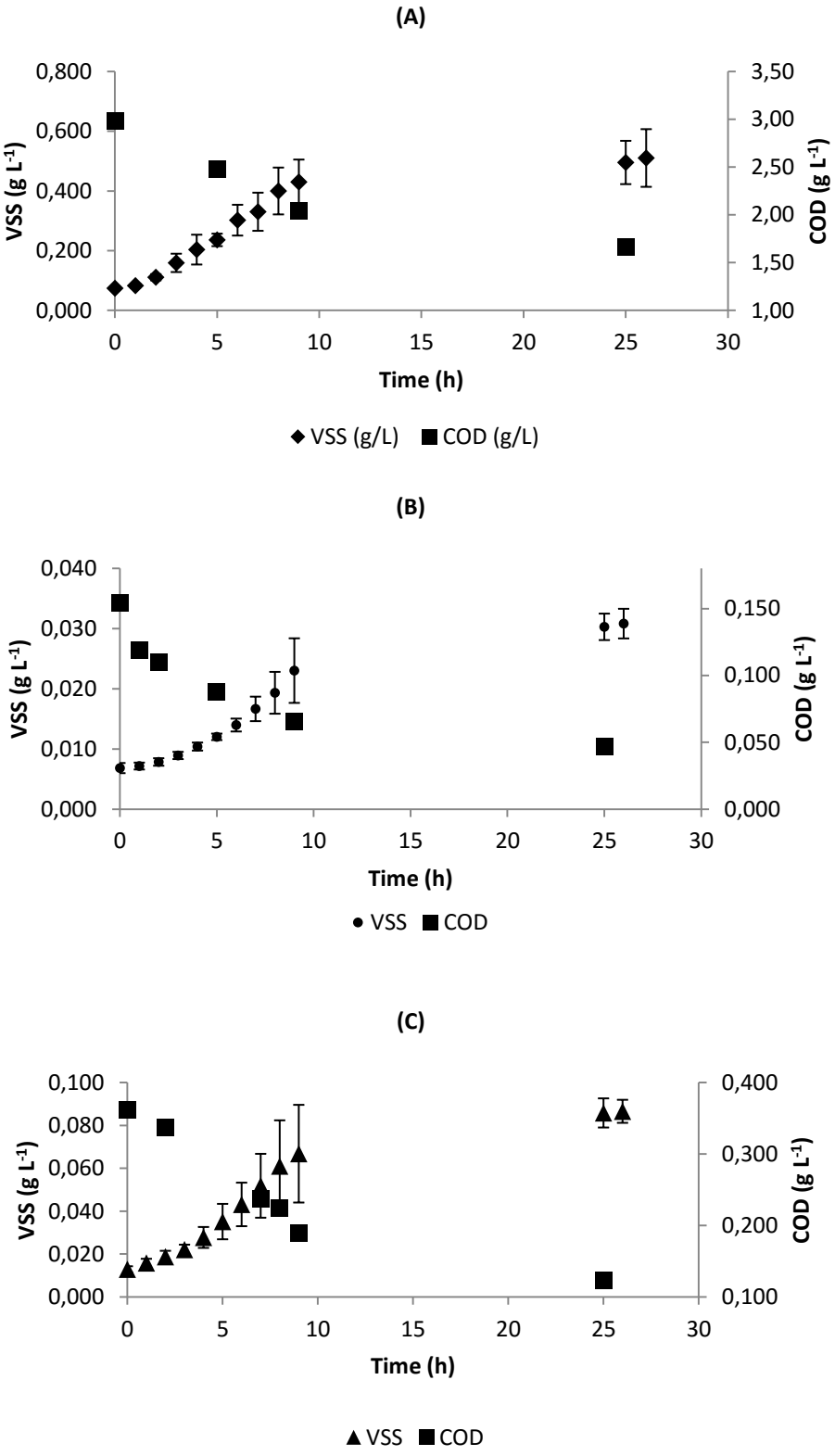

Fig. 3.4. Microbial growth evolution and substrate consumption along the kinetic tests performed with isolate AF1- *Rhodococcus* sp., in growth media with a distinct carbon source: HSSL (A), acetic acid (B) and xylose (C), pH 7 and 20 °C.

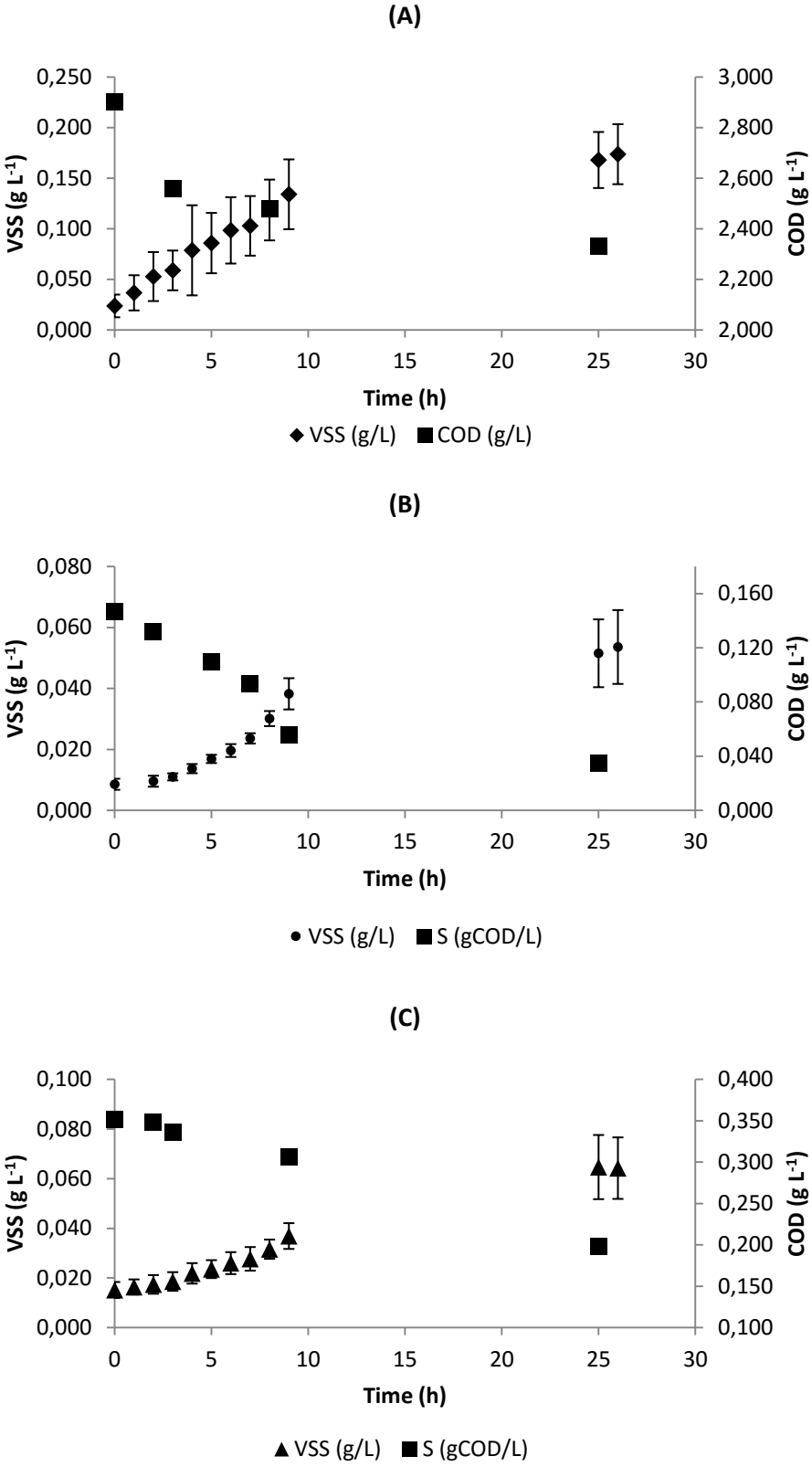

Fig. 3.5. Microbial growth evolution and substrate consumption along the kinetic tests performed with isolate AF2- *Pseudomonas* sp., in growth media with a distinct carbon source: HSSL (A), acetic acid (B) and xylose (C), pH 7 and 20 °C.

a preference in consuming xylose and AF2 (*Pseudomonas* sp.) in acetic acid, as they presented a higher specific substrate consumption rates (q_s) of 0.572 gCOD $gVSS^{-1}$ h^{-1} and 0.430 gCOD $gVSS^{-1}$ h^{-1}, respectively, compared to those with the two other substrates. In future studies, quantification of other components of HSSL over time should be performed for a better understanding of the growth and consumption behavior of both isolates.

A qualitative evaluation of the PHA-accumulating capability of isolates, AF1 and AF2 in liquid medium with three different carbon sources analyzed (HSSL, acetic acid and xylose) was performed (Fig. 3.4, 3.5). Samples taken during the kinetics tests were observed under phase contrast and after Nile blue staining. PHA accumulation by AF1 and AF2 increased during the incubation period reaching a maximum at the late exponential stage of the growth curve. For both isolates, in HSSL and xylose assays, intracellular PHA inclusions were clearly observed. In acetic acid assay, cells were completely fluorescent, which meant that they were completely full of PHA. As PHA accumulation increased and reached the maximum PHA content, some cells of AF1 and AF2 were completely fluorescent in all tests. Nevertheless, the fluorescence intensity was always higher in assays with acetic acid, meaning that cells of isolates AF1 and AF2 were completely full of PHA. This finding was somehow expected when acetic acid was the substrate, since SCOA are the preferred substrates by MMC for PHA production (Gumel et al., 2013). In addition, in assays with HSSL, a higher intensity of fluorescence was clearly observed for AF1 than for AF2, which indicates a higher content of PHA produced by the former. Regarding DS1, this isolate was grown on xylose (Fig. 3.6). This isolate was also able to produce PHA and the increase of inclusion bodies during the growth was clearly observed by Nile blue staining.

The identification of PHA accumulated was performed by GC analysis. The results showed that isolates AF1 and AF2 accumulated a homopolymer P(3HB), and DS1 accumulated a copolymer P(3HB-*co*-3HV), with 4% of 3HV. Many different hydroxy-fatty acids may be converted into polymers, but only if suitable carbon sources are provided as precursor substrates. Some substrates lead to monomer formation of equal carbon chain length. This behavior could explain the copolymer accumulation of the isolate DS1 from

an unrelated carbon source as xylose. Starting from related substrates, the synthesis pathway is closely connected to the fatty-acid β-oxidation cycle (Babel et al., 2001). In some bacteria, the copolymers P(3HB-*co*-3HV) are synthesized from sugars by methyl-malonyl-CoA. Succinyl-CoA is decarboxylated via methyl-malonyl-CoA to propionyl-CoA as the precursor of 3-hydroxyvaleryl-CoA (Valentin and Dennis, 1996).

The isolation of AF1, AF2 and DS1 can contribute to the development of bioprocesses using xylose as substrate. This sugar is the second most abundant in nature, especially in vegetable biomass, but only a few papers have focused on its valorization and even fewer when it comes to PHA production. As xylose is one of the most abundant sugars in nature, it is important to find microorganisms with the capacity to metabolize it and, consequently, convert it into products of interest.

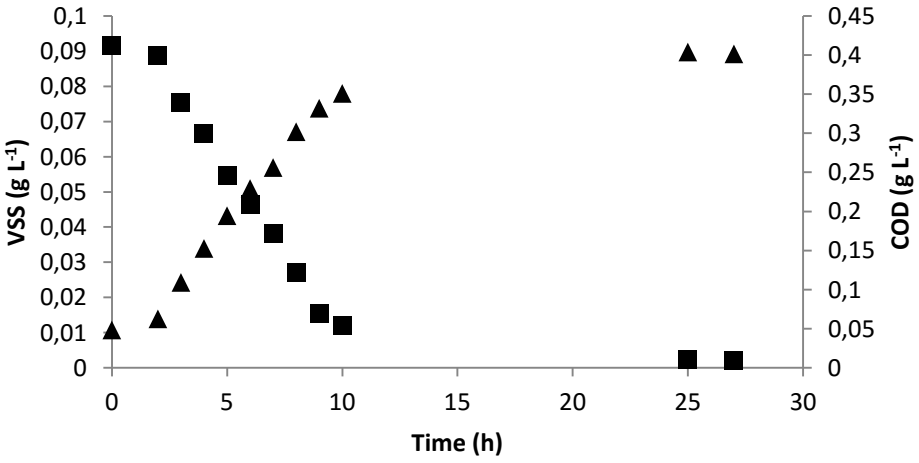

Fig. 3.6. Microbial growth evolution and substrate consumption along the kinetic test performed with isolate DS1- *Klebsiella* sp. with xylose as carbon source

3.3.3. 16S rRNA gene clonal analysis

To identify the unculturable bacteria responsible for PHA accumulation of the MMC, a 16S rRNA gene clonal analysis was performed on DNA extracted from the selected MMC. A total of 31 clones were obtained and 26 identified. Clones were closely related to nine different genera: *Achromobacter* spp., *Comamonas* spp., *Clostridium* spp., *Methylobacillus* spp., *Novosphingobium* spp., *Pedobacter* spp., *Pleomorphomonas* spp., *Pseudomonas* spp. and *Sphingobium* spp. (Table 3.4; Fig. 3.7). Some of the species

highlighted by the clonal analysis have been reported as PHA-accumulating bacteria and have been previously identified or isolated from activated sludge samples, such as *Clostridium* spp. (Emeruwa and Hawirko, 1973), *Comamonas* spp. (Zakaria et al., 2010), *Novosphingobium* spp. (Addison et al., 2007), *Pleomorphomonas* spp. (Xie and Yokota, 2005) and *Sphingobium* spp. (Liang and Lloyd-Jones, 2010). Dai et al. (2015) also investigated the microbial community evolution when fed with poplar hydrolysates. They also observed enrichments in *Alphaproteobacteria* and *Betaproteobacteria* and identified these classes as mainly responsible for P(3HB) accumulation and suggested further isolation of microorganisms to use wood hydrolysates as substrate for PHA production.

Nearly all the identified bacteria belonging to the acclimatized MMC, both from isolation attempts and the 16S rRNA gene clonal analysis, were consistent with the findings of Queirós et al. (2014) after characterization, by FISH, of the acclimatized MMC. *Alphaproteobacteria* was the dominant group of the MMC, accounting for 72.7 ± 4.0% of total bacteria, followed by *Betaproteobacteria*, 11.1 ± 0.37% and *Gammaproteobacteria*, 10.3 ± 0.3%. A minor presence of *Deltaproteobacteria*, *Actinobacteria* and *Bacteroides* was also detected (Queirós et al., 2014). Nevertheless, a complete characterization of all genera selected in the MMC for an optimum design and modeling of the bioreactor is necessary (Dai et al., 2015). This will allow maximum accumulation and productivity by the MMC.

Table 3.4. Representation of the taxonomic affiliations of the clones obtained.

Taxonomic affiliation		N. of clones	Highest similarity	Identity	Clone	Accession number
Achromobacter spp.	*Beta-proteobacteria*	*5*	*A. denitrificans* DSM 30026	99%	FA2	KM873630
Clostridium spp.	*Firmicutes*	*2*	*C.estertheticum* DSM 14864	98%	FA1	KM873629
Comamonas spp.	*Beta-proteobacteria*	*3*	*C. testosteroni* CNB-2	99%*		
Methylobacillus spp.	*Beta-proteobacteria*	*6*	*M. flagellatus* K	99%*		
Novosphingobium spp.	*Alpha-proteobacteria*	*2*	*N.naphthalenivorans* TUT562	96%	FA3 FA4	KM873631 KM873632
Pedobacter spp.	*Bacteroidetes*	*1*	*P. terrae* DS-57	95%	FA6	KM873634
Pleomorphomonas spp.	*Alpha proteobacteria*	*2*	*P.koreensis* Y9	99%*		
Pseudomonas spp.	*Gamma-proteobacteria*	*2*	*P. moraviensis* CCM 7280	99%*		
Sphingobium spp.	*Alpha-proteobacteria*	*3*	*Sphingobium* SYK-6	95%	FA5	KM873633

3.4. Conclusion

Isolates belonging to four of the six groups of the microbial community identified previously were isolated and characterized. It was possible to successfully isolate *Rhodococcus* sp., *Pseudomonas* spp. and *Klebsiella* sp. The isolates could both grow and accumulate PHA despite the harsh conditions brought about by complex substrates, such as HSSL, as well as to use xylose as the carbon source. Moreover, from the clonal analysis, the identification of *Novosphingobium* spp., *Sphingobium* spp. and *Pleomorphomonas* spp. were possible, they having been previously found to be related to PHA production.

This work constitutes a step forward towards the identification of the microorganisms responsible for the accumulation of PHA in the MMC, and, consequently, to the valorization of HSSL as a complex substrate for biological processes. The isolation of microbial strains able to use xylose, the second most abundant sugar in nature, for PHA

production could be an important advance leading for the valorization of raw materials resulting from the bioprocessing of vegetable biomass.

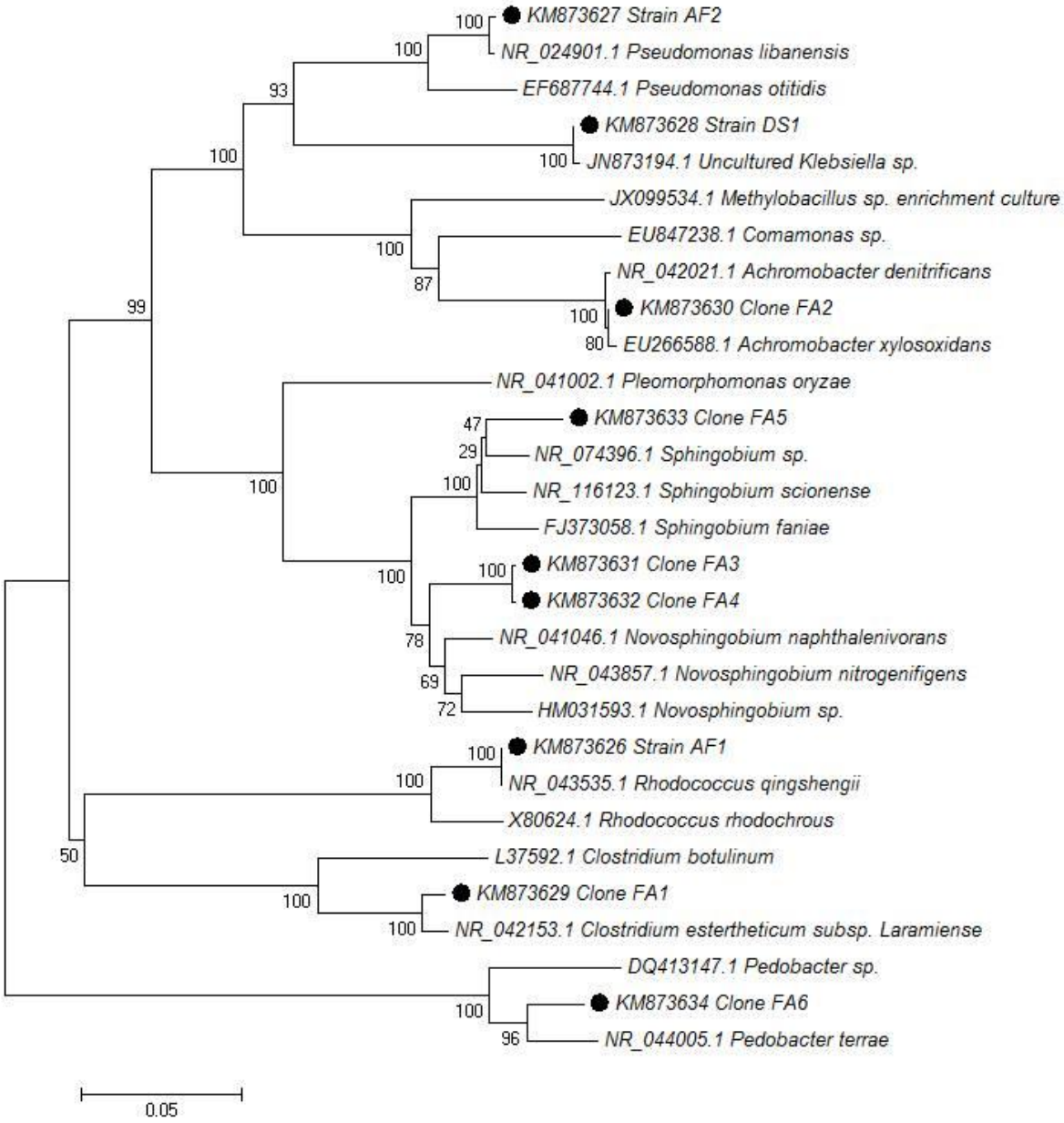

Fig. 3.7. Phylogenetic tree. Based on full-length nucleotide sequences of 16S rRNA gene of isolates AF1, AF2 and DS1 and clones FA1, FA2, FA3, FA4, FA5 and FA6 (in the tree preceded by a filled dot). The evolutionary history was inferred using the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA6.

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Chapter 4

Long-term operation of a two-stage PHA production process from HSSL

The development of sustainable industrial processes demands the recovery and transformation of their by-products. The production of PHA by MMC is one of the available routes. This study evaluated the possibility of applying a two-step PHA production process using an industrial by-product, HSSL, as feedstock for an MMC. The first step consisted in the selection of PHA-storing microorganisms using an aerobic dynamic feeding strategy followed by a second step, accumulation.

The selected MMC, despite not being able to use the main carbon source of HSSL, lignosulphonates, revealed the capacity to consume acetic acid and xylose to accumulate P(3HB-*co*-3HV) and glucose biopolymer. The maximum PHA content was relatively low, 6.6%, with a maximum production yield of 0.49 CmmolHA $CmmolS^{-1}$ during the selection stage.

An MMC could adapt to HSSL by consuming acetic acid and xylose. The imposed operational conditions resulted in a partial selection of the culture, since only acetic acid consuming organisms experienced a real feast/famine regime, resulting in low PHA production.

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4.1. Introduction

PHA display a high replacement potential over conventional plastics due to their wide range of thermoplastic properties (Laycock et al., 2013). Moreover, PHA are fully biodegradable and can be produced from renewable sources, offering a possible solution to the environmental hazards displayed by conventional plastics (Crank and Patel, 2005).

The industrial established PHA production processes are based on the use of recombinant organisms and refined substrates, resulting in an expensive final product when compared to petroleum-derived plastics (Chanprateep, 2010; Chen, 2009). The use of MMC and surplus-based feedstocks is being investigated as an alternative way to reduce the overall production costs. The process layout usually comprises three different steps such as AF of the surplus-based feedstocks, selection of PHA-storing microorganisms, and PHA accumulation (Dionisi et al., 2004). To enhance PHA production, an MMC should be steered through the operation parameters imposed, to maintain efficient PHA-storing organisms that will overgrow the remaining population.

In the last years, several studies looked for the possibility of implementing evolutionary engineering on PHA-storing communities based on synthetic wastewaters. Type and concentration of substrate, SRT, HRT, OLR, cycle length, C/N ratio, pH, temperature, reactor operation mode, among others, are some of the parameters that governed the selective pressure imposed to select PHA-storing communities (Reis et al., 2011). In fact, it was already possible to select a stable microbial community for PHA production able to reach 89% cdw in the accumulation step, a value comparable to those obtained with pure cultures in terms of maximum PHA content (Johnson et al., 2009).

The type of substrate should also be taken into account, since by using surplus-based feedstocks PHA production costs can be reduced by 50% (Serafim et al., 2008). A considerable number of works already began seeking for processes based on using these substrates, namely olive mill wastewaters (Dionisi et al., 2005), paper mill wastewaters (Bengtsson et al., 2008; Jiang et al., 2012), pulp and paper mill by-products (Queirós et al., 2014), palm oil mill effluents (Gobi and Vadivelu, 2014; Mohd et al., 2012), pyrolysis by-product (Moita and Lemos, 2012), sugar cane molasses (Albuquerque et al., 2007), crude glycerol (Moita et al., 2014), fermented waste activated sludge (Chen et al., 2014;

Morgan-Sagastume et al., 2010) or food processing wastewaters (Anterrieu et al., 2014; Khumwanich et al., 2014; Liu et al., 2008). The choice of the right surplus-based feedstock should take into consideration its chemical composition since it influences the monomeric composition of PHA, which determines the physical and mechanical properties of the final polymer. Sometimes, to increase the amount of SCOA present, the preferred substrate of MMC for PHA production, the feedstock should be submitted to AF. It was already demonstrated that PHA production by MMC can be manipulated to produce copolymers with different monomeric composition by changing the amount of SCOA using synthetic wastewaters (Jiang et al., 2011; Wang et al., 2013) or by controlling the operational conditions of the acidogenic reactor (Albuquerque et al., 2011).

Queirós et al. (2014) used for the first time HSSL, a by-product of pulp industry, as substrate for PHA production by an MMC, reaching a content of 67.6% in the accumulation step. However, a complete steady-state was not reached on the reactor and instability of PHA and biomass production were observed (Queirós et al., 2014). Therefore, the objective of this study was to infer the possibility to establish a steady process with a robust microbial community able to deal with the fluctuations of the HSSL composition. As the HSSL is rich in acetic acid, the two-step process was chosen to further decrease the operational costs.

4.2. Materials and Methods

4.2.1. Culture Medium

HSSL from magnesium based acidic sulfite pulping of *Eucalyptus globulus* was supplied by Caima – Indústria de Celulose S.A. (Constância, Portugal). Pre-evaporated HSSL was collected from an inlet evaporator of a set of multiple-effect evaporators to avoid the presence of free SO_2. To remove part of the most recalcitrant compounds, HSSL was submitted to a preliminary pretreatment (Queirós et al., 2014). The pretreatment started with a pH adjustment to 7.0 with 6 M KOH, followed by aeration with compressed air (6 h per liter – 6 h L^{-1}). Then, the liquor was centrifuged for 1 h at 5000 rpm. The precipitated colloids were filtered off using a 1 µm glass microfiber filter. Finally, the total COD of pretreated HSSL was determined (≈ 200 gCOD L^{-1}). Lignosulphonates (LS) were still

the main constituents (120 – 160 g L^{-1}) along with xylose and acetic acid (≈ 50 and 18 g L^{-1}, respectively). No phosphates and ammonia were detected in HSSL (Table 4.1).

Table 4.1. HSSL composition.

	Concentration (g L^{-1})	
Components	**Queirós et al. (2014)**	**This work**
Lignosulphonates	78.2 ± 0.6	160 – 180
Acetic Acid	8.2 ± 0.3	17 – 24
D-Xylose	24.6 ± 0.5	49 – 53

To achieve an OLR of 17 gCOD L^{-1} d^{-1} in the SBR, HSSL was diluted with a mineral solution (1:17). The mineral solution was composed by (per liter of distilled water): 160 mg $MgSO_4 \cdot 7H_2O$, 80 mg $CaSO_4 \cdot 2H_2O$, 20 mg $FeCl_3$, 8 mg $Na_2MoO_4 \cdot 2H_2O$, 160 mg NH_4Cl. The pH of the medium was adjusted to 7.0 and the medium was autoclaved for 20 min at 121 °C. Under sterile conditions KH_2PO_4 (16 mg L^{-1}) and K_2HPO_4 (64 mg L^{-1}) were added to the medium. Thiourea (400 mg L^{-1}) was also added to inhibit nitrification.

4.2.2. Reactor Operation

A SBR was inoculated with activated sludge obtained from the aerobic tank of the municipal wastewater treatment plant Aveiro Norte (SIMRia). The SBR working volume was 1.5 L and it was operated under ADF conditions, during which alternating feast and famine phases were imposed. The SBR was operated for 430 days, in cycles of 8 h that comprised 7 h of aerobiosis, with fresh medium supplied during the first 0.25 h, 0.5 h of settling (with agitation and aeration switched off) and 0.25 h of withdrawing (one third of the reactor volume was removed) resulting in a HRT of 1 day. An SRT of 5 days was imposed by purging 100 mL at end of the aerobic period. Reactor stirring (400 rpm), aeration and feeding and withdrawing pumps were controlled with timers. DO and temperature were measured with Oxygen Meter Transmitter M300 (Mettler-Toledo Thornton, Inc). The system worked without pH and temperature control, although their values were monitored. To prevent foam formation, diluted silicone anti-foam (1:20) was

manually added when excessive foam was observed. SBR was cleaned daily to prevent excessive biofilm formation on reactor walls and electrodes surfaces.

SBR cycles were monitored periodically by taking samples across the entire reaction period and acetic acid, xylose, LS, ammonium, COD, PHA and glucose biopolymer (GB) content were analyzed.

4.2.3. Accumulation Assay

The assay was performed in the SBR itself by adding feed to the system in a pulse-wise manner to avoid potential substrate inhibition. The decision of adding a new pulse was based on the DO profile. Once the carbon was depleted and the DO increased abruptly, aeration and stirring were turned off allowing biomass to settle, followed by medium replacement. The feeding had the same composition and it was in the same proportion as for the daily operation. Samples were collected every 15 min along with the pH and DO values.

4.2.4. Analytical Methods

Biomass concentration was determined using total suspended solids (TSS) and volatile suspended solids (VSS) procedure described in *Standard Methods* (Clesceri et al., 1998).

PHA quantification and monomeric composition was determined using gas chromatography following the procedure described by Moita et al. (2014)

GB was determined after being extracted from lyophilized cells through acidic digestion (1 mL HCl 0.6 M, 2 h, 100 °C). Digested samples were filtered; the liquid fraction was analyzed by HPLC using an Aminex HPX-87 H column (Bio-Rad Laboratories, CA, USA), at 60 °C, and a Refractive Index detector (Merck, Germany), using H_2SO_4 0.01 N as eluent (0.5 mL min^{-1}).

COD was measured accordingly to Standard Methods. Acetic acid and xylose were measured by HPLC following the same procedure described by Queirós et al. (2014). LS was measured per Restolho et al. (2009). The absorbance of samples was measured in a spectrophotometer at 273 nm, after a dilution of 1:200. LS concentration was calculated

using the Beer-Lambert law with a molar attenuation coefficient of 7.41 g^{-1} cm^{-1} (Restolho et al., 2009; Xavier et al., 2010).

Ammonium concentration was followed using a Thermo Scientific Ion Selective Electrode after adding 20 µL of Ionic Strength Adjuster (ISA) to 1 mL of sample and applying a calibration curve obtained with standard solutions of NH_4Cl.

4.2.5. Calculations

PHA and GB contents were calculated as a percentage of TSS on a mass basis:

$$\%\ \mathrm{PHA} = \frac{\mathrm{gHA}}{\mathrm{gTSS}} \times 100 \qquad (4.1)$$

$$\%\ \mathrm{GB} = \frac{\mathrm{gGB}}{\mathrm{gTSS}} \times 100 \qquad (4.2)$$

Active biomass (X) was obtained by subtracting the storage products, PHA and GB, from VSS as (in g L^{-1}):

$$\mathrm{X} = \mathrm{VSS} - \mathrm{PHA} - \mathrm{GB} \qquad (4.3)$$

Feast to Famine ratio (F/F) was calculated dividing the time needed to consume acetic acid by the remaining time of the cycle (Serafim et al., 2004).

It was assumed that all ammonia was consumed for growth considering that thiourea fully inhibits nitrification. Active biomass elemental composition was represented by the molecular formula $C_5H_7NO_2$ indicating that 1 mg of N is needed to produce 8 mg of active biomass.

Xylose specific consumption rate ($-q_{Xyl}$), acetic acid specific consumption rate ($-q_{Acet}$), PHA specific production rate (q_{PHA}) and GB specific production rate (q_{pGB}) were determined by adjusting linear functions to the experimental data for each variable concentration divided by the biomass concentration at that point along time, and calculating the first derivative at time zero. PHA production yield on substrate ($Y_{HA/S}$), GB production yield on substrate ($Y_{GB/S}$) and biomass production yield on substrate ($Y_{X/S}$)

were calculated by dividing the amount of each parameter by the total amount of substrate consumed (corresponding to the sum of acetic acid and xylose concentrations). PHA production yield on acetic acid ($Y_{HA/Acet}$) and GB production yield on acetic acid ($Y_{GB/Acet}$) were calculated by dividing the amount of each parameter by the total amount of acetic acid consumed.

4.3. Results and Discussion

4.3.1. SBR Operation

To achieve an MMC with a good PHA-storing capacity adapted to a complex substrate such as HSSL, a SBR fed with this feedstock was operated continuously for 430 days under ADF conditions. Along the operational period several parameters were monitored: COD, acetic acid, xylose and ammonia consumption; PHA and biomass production. These parameters allowed evaluating the MMC acclimatization to the operational conditions and its PHA production capacity.

Generally, F/F ratio is considered a good indicator to assess the stability of the SBR (Reis et al., 2011; Valentino et al., 2014). This ratio is determined by using DO in the medium as an indicator, since usually when the exhaustion of the carbon source occurs, an abrupt increase on this value is verified. This allows identifying the transition between the two phases and determining their length in an easy and practical way. Since on-line data acquisition of DO concentration values were not available on this system, F/F ratio values were determined based on the DO values registered at the time of samples collection in the daily cycle analysis. The F/F ratio evolution along the operational period of the SBR is shown in Fig. 4.1. The MMC took more than 250 days to reach a more stable operation with F/F ratio values around 0.5, the range during which storage response is expected (Reis et al., 2011). After 230 days, F/F ratio values remained stable, however considerably high. On day 261, they dropped to 0.25, being stable until day 331 when F/F ratio values decreased now to 0.125 remaining constant until the end of reactor operation. Usually, F/F ratio can be varied in the SBR by changing the OLR. Different studies showed that a low OLR resulted in low F/F ratios and, consequently, favored a selective pressure for PHA-storing microorganisms. For this reason, low values of OLR are

generally used to operate the selection stage (Johnson et al., 2009; Serafim et al., 2004). Some authors found that F/F ratio values up to 0.26 resulted in a high storage response; while, for higher ratios, a very unstable storage or, predominantly, growth responses were obtained (Albuquerque et al., 2010; Dionisi et al., 2006). The F/F ratio values of 0.25 and 0.125 obtained during the more stable operating period of the SBR were comparable to those described in the literature to be associated with a predominantly storage response. However, it is important to stress that, despite the high OLR imposed in the SBR, only a small part corresponded to acetic acid, the main carbon source for PHA production. For this reason, F/F ratios were calculated based only on the exhaustion of acetic acid, related with sudden increase of DO values.

Other parameters were also used to access SBR performance of the selected MMC such as consumption rates and production yields. The HSSL used in this work had a different composition from the one tested by Queirós et al. (2014) Changes in the pulping process at Caima – Indústria de Celulose S.A. led to a different composition of HSSL (Table 4.1). Although the HSSL used in the present work was more concentrated in organic compounds, the relative proportion of LS, acetic acid, and xylose, 8.30:1.0:2.5 (based on average values), did not differ much from that tested by Queirós et al. (2014) with a ratio of 9.5:1.0:3.0. Contrary to what was observed by Queirós et al. (2014), regarding LS, a clear tendency along SBR cycles was not observed; thus, not revealing a significant consumption (Table 4.2). Concerning acetic acid and xylose, the results of substrate uptake rates did not reach stable values along the reactor operation. The results showed that xylose was preferably consumed until 310th day of operation and its consumption rate increased from the beginning of operation until the maximum value on day 270 (9.81 Cmmol L^{-1} h^{-1}). After this day, a consistent decrease was verified until reaching the value of 4.10 Cmmol L^{-1} h^{-1} at the end of operation. On the other hand, acetic acid, after starting with a low consumption rate, 0.61 Cmmol L^{-1} h^{-1}, evolved along the operational period with constant growth even surpassing xylose as preferable substrate after day 312 and reached a maximum value of 7.78 Cmmol L^{-1} h^{-1} on day 332. During the period with a F/F ratio of 0.125, the preference shown by the culture towards the main substrates switched back, with xylose being faster consumed than acetic acid, on day 339.

MMC are known to preferentially consume SCOA and to not use sugars such as xylose and glucose for PHA production, as reported by many authors (Carta et al., 2001; Dircks et al., 2001). Instead, carbohydrate consumption is associated with glycogen storage since it is more energetically favorable than PHA storage (Dircks et al., 2001). Indeed, as observed by Carta et al. (2001), in the presence of sugars and acetic acid, the latter was stored as P(3HB) whereas the former, such as glucose or starch, were stored as glycogen. However, Queirós et al. (2014) observed PHA accumulation when xylose was supplied as a sole carbon source. Later, Ferreira et al. (2016) were able to isolate microorganisms, from the same MMC, which were able to consume xylose and store PHA.

Despite the existence of bacteria able to convert xylose into PHA, probably their presence in the selected MMC was residual, and, consequently, acetic acid consumption rate increased along the SBR operational time showing that the culture enrichment occurred for organisms that preferably consume this substrate. The presence of bacteria able to convert sugars into PHA in MMC that use SCOA is quite frequent. As an example, Dionisi et al. (2006) detected the presence of species belonging to *Alcaligenes* genus, usually associated with PHA production from sucrose, in a PHA-storing MMC fed with a mixture of acetic, propionic and lactic acids.

The SBR reached an apparent steady state after 270 days of operation, based on the stability of the F/F, once %PHA stabilized earlier. In this work a considerable longer time when comparing with the range of values described in the literature, usually from 30 to 167 days, was attained (Albuquerque et al., 2010; Moita and Lemos, 2012). Although using HSSL with a different composition for PHA production by MMC, Queirós et al. (2014) also reported culture instability. The need for a longer time of operation to reach a steady state could be due to the high content of microbial inhibitors of HSSL. As reviewed by Pereira et al. (2013), HSSL contains compounds such as gallic acid, pyrogallol and furfural that are known to have a toxic effect on bacteria and, consequently, could affect the production of active biomass and PHA.

Table 4.2. Comparison between the MMC described in the present study and in Queirós et al. (2014).

Study	Dissolved oxygen	Acetic acid consumption	Xylose consumption	LS consumption	Type of PHA produced	% max PHA (batch HSSL)	Culture Selection
This study	Increased sharply with acetic acid exhaustion	Fully consumed	Fully consumed after 340 days of operation	No clear tendency	P(3HB-*co*-3HV), 20% 3HV	4. 6%	Low PHA accumulation
Queirós et al.	Increased sharply with acetic acid exhaustion	Fully consumed	Around 30% was consumed	Around 14% was consumed	P(3HB)	60.2%	High PHA accumulation [a]

Nevertheless, the obtained results showed that, despite the presence of inhibitors, a PHA-storing MMC with tolerance to these compounds could adapt to the imposed conditions on the SBR and to use at least some of the components of HSSL as substrate. This population was able to maintain an average value of active biomass of 2.85 ± 0.88 g L^{-1} and a relatively low but stable PHA content of 4.31 ± 1.18%, along the operational time (Fig 4.1).

The highest PHA content obtained during the selection process was 6.66% on day 237 but the highest PHA storage yield occurred on day 270, 0.49 CmmolHA CmmolS^{-1} or 0.45 gCODHA gCOD^{-1}. The PHA content was slightly lower than the range, 9 – 10%, observed for the selection step of other reported works (Albuquerque et al., 2007; Moita and Lemos, 2012). However, the highest storage yield obtained in the present work was in the range of the values obtained in similar accumulation assays found in the literature, 0.24 – 1 gCODHA gCOD^{-1} (Moita et al., 2014).

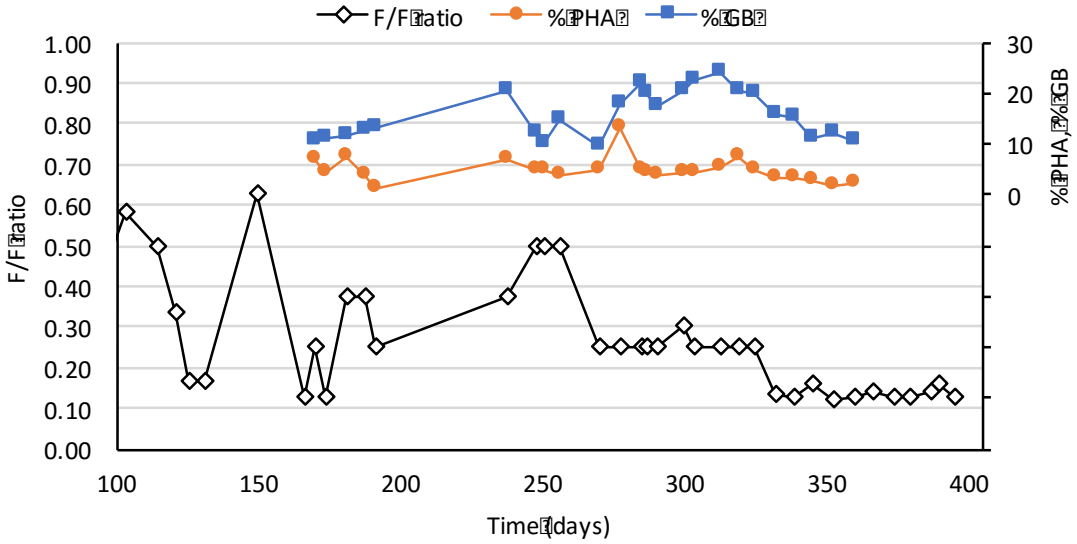

Fig 4.1. F/F ratio, PHA and GC contents evolution along the reactor operation.

Regarding polymer composition, the MMC produced a copolymer of P(3HB-*co*-3HV), with 20% of 3HV. Since acetic acid is considered the main precursor to produce 3HB, other components of HSSL were probably used for 3HV formation, like xylose as observed by Ferreira et al. (2016) with one of their isolates. Another possible explanation

was the conversion of GB to propionyl-CoA, one of the precursors of 3HV formation that could occur if very low DO was present (Serafim et al., 2008). Different from what was observed by Queirós et al. (2014), where a homopolymer of P(3HB) was produced from HSSL, the production of P(3HB-*co*-3HV) in this work is particularly valuable since it is industrially more appealing (Laycock et al., 2013; Queirós et al., 2014).

Considering the high sugar content of HSSL, a significant but variable GB content was obtained, 18.32 ± 4.14%, reaching a maximum value of 24.21% on the 312th day (Fig 4.1). Thus, when compared with PHA storage yield, a higher GB storage yield was generally obtained.

Since biomass growth and polymer accumulation usually compete for the carbon sources it would be expected to establish a relationship between them. However, according to experimental data, there was not a clear tendency between such parameters. While active biomass remained stable after day 260 (data not shown), polymer accumulation yields showed different tendencies: PHA storage yield seemed to decrease, while GB accumulation had an increased tendency but both in a non-consistent way. This might be due to the complexity of the raw material used as substrate and the different bacterial groups present in the culture, which consumed the different carbon sources without competition and at different rates (Moita and Lemos, 2012). Although daily cycles analysis demonstrated an MMC preference for substrate storage as reserve polymers, substantial enrichment of a PHA accumulating culture was not verified in comparison to Queirós et al. (2014) Despite the use of similar substrates but with different compositions, the selected MMC showed different behaviors (Table 4.2).

4.3.2. SBR cycles

The SBR was operated continuously under ADF conditions for 430 days, during which successive 8 h cycles were followed. Along that period, the SBR was characterized through monitoring of individual cycles that allowed assessing the system performance. Fig. 4.2 shows a representative cycle with a F/F ratio of 0.25 on day 319 of operation.

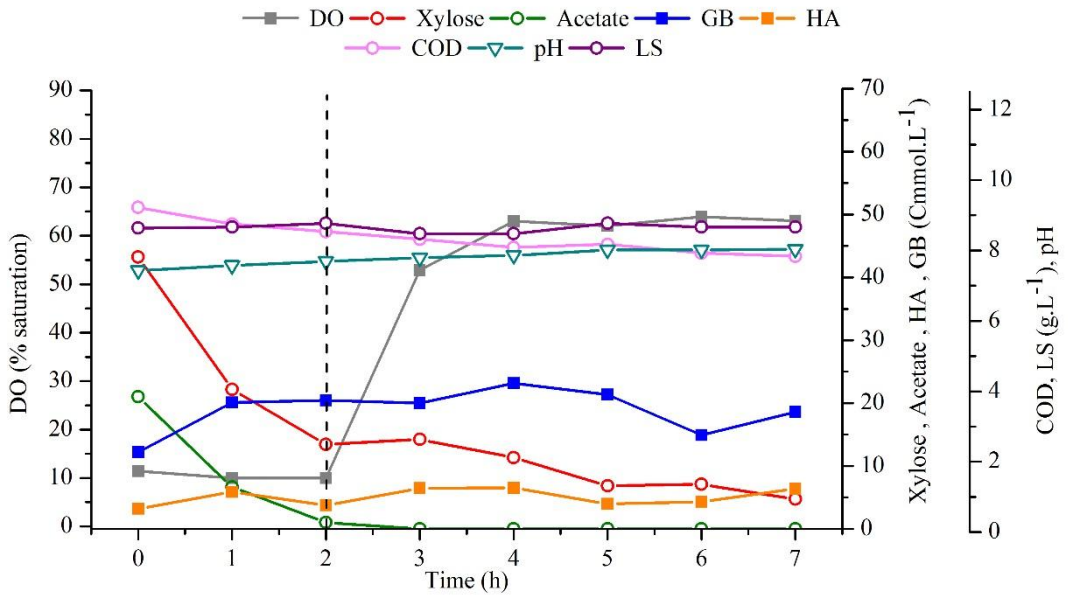

Fig 4.2. Representative cycle monitored on 319th day of operation. F/F ratio of 0.25.

Since pH was not controlled in the SBR, its profile varied along the cycle. As expected, pH increased along the cycle from 7.40 until 8.03. The pH variation observed was lower than the pH variation between 8.0 and 10.0 observed by Serafim et al. (2004), in a SBR fed with acetic acid without pH control. Queirós et al. (2014) hypothesized a possible buffer behavior of HSSL. LS consumption exhibited a variable behavior between cycles and although a slight consumption seemed to occur along this cycle, there was not a clear tendency and consumption rates could not be determined (Fig 4.2). Even though LS consumption was not significant, the microbial community seemed to tolerate the presence of such components, thus not showing a strong inhibitory effect. Xylose and acetic acid were both consumed along the cycle, although at different rates. While acetic acid was totally consumed after 2 h at a rate of 0.12 CmmolAcet CmmolX^{-1} h^{-1}, xylose, due to its higher concentration, was not fully exhausted by the end of the cycle. Xylose uptake rate was not constant through the whole cycle: it was higher until acetic acid exhaustion, 0.18 CmmolXyl CmmolX^{-1} h^{-1}, decreasing to 0.029 CmmolXyl CmmolX^{-1} h^{-1} afterwards. Similar profiles for carbon sources were observed by Moita et al. (2014) when using crude glycerol to select a PHA-storing MMC concerning glycerol and methanol, respectively (Moita et al., 2014). As in Queirós et al. (2014), the consumption of the xylose without being exhausted was also observed.

The evolution of external carbon sources concentration is usually reported to be correlated with DO concentration in many ADF systems described in the literature (Dionisi et al., 2005; Moita et al., 2014; Queirós et al., 2014). The time of the exhaustion of the carbon source usually corresponds to an abrupt increase of DO in the medium, but in the present work DO only reacted to acetic acid. Since the carbon sources were consumed in different ways, the definition of the feast and famine cannot be applied to all the different microorganisms in the culture. For this reason, 2 h were considered the duration of the feast phase, while 6 h were accounted for the famine phase according to acetic acid and oxygen consumption profiles. Other than acetic acid, the large variety of carbon sources that existed in HSSL were not depleted along the entire cycle as shown by COD, allowing diverse microbial populations to co-exist in the system, which could be an explanation for the long period needed for the SBR to reach a pseudo steady-state (PSS). Consequently, populations without the ability to store polymers could grow and persist in the SBR throughout the consumption of such carbon sources. In fact, the sugar fraction of HSSL, namely xylose, was continuously consumed during the entire cycle and, together with LS, could be one of the possible substrates used by the microbial population unable to accumulate polymers. In this way, there was an impairment of the selection purpose of the ADF conditions, by decreasing the selective pressure imposed. The presence of this side population could justify the low PHA storage contents observed along the SBR operational period. Moita and Lemos (2012) observed the same culture behavior using bio-oil resultant from pyrolysis process which contained organic acid and sugar fractions as well as other recalcitrant compounds.

Regarding PHA storage, accumulation occurred at a specific rate of 0.032 CmmolHA $CmmolX^{-1}$ h^{-1}, corresponding to 0.030 gCODHA $gCODX^{-1}$ h^{-1}, until reaching a maximum content of PHA, 3.18%, when acetic acid was depleted. The specific PHA accumulation rate obtained was in the range of the reported in the literature, 0.0082 – 0.42 gCODHA $gCODX^{-1}$ h^{-1}, but not for the PHA storage yield 0.064 CmmolHA $CmmolS^{-1}$ ($Y_{HA/S}$ = 0.064 gCODHA $gSCOD^{-1}$), 0.08 – 1 gCODHA $gSCOD^{-1}$ (Serafim et al., 2008). Since acetic acid can be considered the main precursor for PHA formation, an improvement on PHA storage yields could be observed when only acetic acid was accounted (Table 4.3).

Consequently, now the $Y_{HAs/Acet}$ falls into the mentioned range, 0.17 CmmolHA CmmolAcet^{-1} (equivalent to 0.16 gCODHA gCODAcet^{-1}). This was expected as xylose consumption was also considered for PHA yield calculation and probably it did not contribute to its accumulation. To clarify the contribution of each carbon source to polymer accumulation, batch tests should have been done with each substrate individually.

Xylose was probably deviated for GB production during the feast phase. Comparing the specific production rates of the two biopolymers produced, GB synthesis, 0.095 CmmolGB CmmolX^{-1} h^{-1}, was more than three times faster than PHA storage. The maximum GB content coincided with the PHA maximum although being significantly higher, 15.6%. Also, GB storage yield, 0.21 CmmolGB CmmolS^{-1}, was higher than the PHA storage yield. These results were consistent with the findings by Dircks et al. (2001), who showed that glycogen storage was faster than PHA production and more efficient in terms of ATP than PHA. After reaching their maximum value, the amount of PHA and GB decreased during the famine phase due to its consumption by bacteria for their maintenance and growth, although xylose and LS were still present and being consumed. This consumption was probably due to the side population that remained in the system and do not contribute to the biopolymers production.

By day 331 of operation, the F/F ratio decrease to 0.125, suggesting that the culture selection did not ended when a stable F/F of 0.25 was observed. A representative cycle of this period (day 400) is shown in Fig. 4.3.

Similar profiles as in day 319 were observed but with faster consumption of acetic acid, 0.29 CmmolAcet CmmolX^{-1} h^{-1}, and xylose, 0.31 CmmolXyl CmmolX^{-1} h^{-1}. Xylose consumption decreased again after acetic acid depletion to 0.08 CmmolXyl CmmolX^{-1} h^{-1}. Despite the decrease in F/F ratio, the amount of PHA produced remained in the same range compared to the previous cycle presented (Table 4.3). PHA content was 5.1%, being quickly accumulated at 0.031 CmmolHA CmmolX^{-1} h^{-1}. In this phase, $Y_{HAs/S}$ presented a value of 0.10 CmmolHA CmmolS^{-1}, a value inside the range previously shown, and higher when acetic acid was the only substrate considered, $Y_{HAs/S}$ = 0.19 CmmolHA CmmolAcet^{-1}. Unfortunately, GB was not possible to monitor along this cycle.

Table 4.3. Kinetic and stoichiometric parameters obtained for the SBR and Batch test.

Assay		$-q_{Acet}$	$-q_{Xyl}$	q_{pHA}	q_{pGB}	$\%HA_{max}$	$\%GB_{max}$	X	$Y_{HA/S}$	$Y_{HA/Acet}$	$Y_{GB/S}$	$Y_{GB/Acet}$
Cycle 319		0.12 ± 0.35 (3)	0.18 ± 0.33	0.032 (3)	0.095 (3)	3.18	15.56	2.32	0.064	0.17	0.21	0.55
Cycle 400		0.29 ± 0.046 (3)	0.31 ± 0.0064	0.037 ± 0.048 (3)	nd	5.1	nd	3.15	0.10	0.19	nd	nd
Batch test	**1º pulse**	0.017 ± 0.057 (7)	0.071 ± 0.057	0.11 ± 0.22 (7)	nd	3.11	nd	3.19	0.11	0.58	0.09	nd
	2º pulse	0.016 ± 0.012 (6)	0.057 ± 0.093	0.07834 ± 0.12 (6)	nd	3.73	nd		0.10	0.51	0.04	nd
	3º pulse	0.017 ± 0.082 (6)	0.010 ± 0.040	0.01 ± 0.16 (6)	nd	4.65	nd		0.14	0.75	0.10	nd

nd – not determined

$-q_{Acet}$ (CmmolAcet CmmolX^{-1} h^{-1}), $-q_{Xyl}$ (CmmolXyl CmmolX^{-1} h^{-1}), q_{pHA} (CmmolHA CmmolX^{-1} h^{-1}), q_{pGB} (CmmolGB CmmolX^{-1} h^{-1}), $\%HA_{max}$ (% gHA gTSS^{-1}), $\%GB_{max}$ (% gGluc gTSS^{-1}), X (g L^{-1}), $Y_{HA/S}$ (CmmolHA CmmolS^{-1}), $Y_{HA/Acet}$ (CmmolHA CmmolAcet^{-1}), $Y_{GB/S}$ (CmmolGluc CmmolS^{-1}), $Y_{GB/Acet}$ (CmmolGluc CmmolAcet^{-1}), $Y_{X/S}$ (CmmolX CmmolS^{-1})

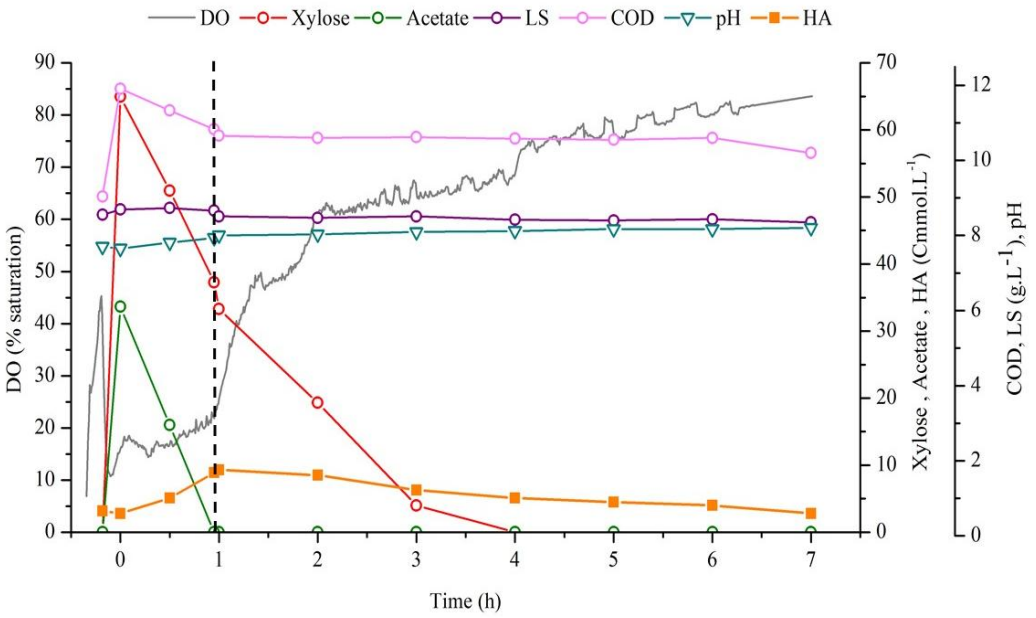

Fig 4.3. Representative cycle monitored on 400th day of operation. F/F ratio of 0.125.

4.3.3. Batch Test

The next step of a two-stage PHA production process is accumulation. Fig 4.4 shows the results of the accumulation test performed in fed batch mode with the addition of three feed pulses. PHA were produced at a similar rate along the three pulses reaching an average specific PHA accumulation rate of 0.094 ± 8.3×10^{-3} CmmolHA CmmolX^{-1} h^{-1} and a storage yield of 0.12 ± 0.021 CmmolHA CmmolS^{-1}. Kinetic parameters for each pulse are listed in Table 4.3.

The maximum PHA content achieved, 4.6%, at the end of the third pulse was substantially lower than other works that used real complex substrates, which ranged from 25 to 77% (Albuquerque et al., 2010; Jiang et al., 2012; Moita et al., 2014). The low PHA storage of the culture may be due to the low acetic acid content of the HSSL, which was probably the preferential carbon source for PHA production by the selected MMC. Moreover, the presence of microorganisms unable to accumulate PHA in the system that might be responsible for the consumption of the sugar-based compounds registered during the entire assay, Fig. 4.4, could also contribute to the low PHA storage content. As seen in Table 4.3, the storage yield improved significantly when acetic acid was considered the only substrate used for PHA production and, for this reason, the

conversion of xylose via a pre-fermentation step would be the logical sequence to improve this process. In this test, only three pulses of substrate were added. Considering that in the end of the third pulse the culture was still able to accumulate PHA and consume the substrates without any sign of inhibition, more pulses should have been added to improve the PHA storage content. Also, future accumulation tests should be performed with limiting conditions of ammonium and oxygen to channel the carbon consumed to polymer accumulation and minimize biomass growth.

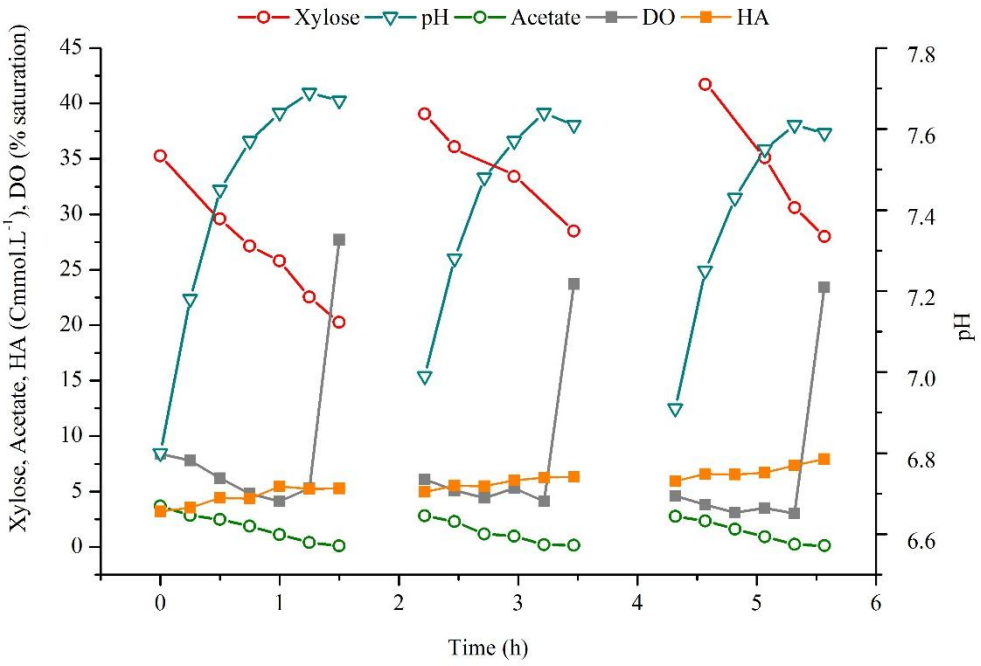

Fig. 4.4. PHA accumulation assay performed at 293rd day of operation.

4.4. Conclusion

An MMC could adapt to HSSL, by consuming acetic acid and xylose but not the main carbon source, lignosulphonates. Besides PHA, GB accumulation was also observed. The imposed operational conditions resulted in a partial selection of the culture, since only acetic acid consuming organisms experienced a real feast/famine regime, while xylose remained present almost through the entire cycle. Consequently, the resulting culture turned out to be more specialized in the production of GB instead of PHA. During the selection stage the average contents of 18.32 ± 4.14% and 4.31 ± 1.18% for GB and

PHA and average production yields of 0.34 ± 0.12 CmmolGluc $CmmolS^{-1}$ and 0.20 ± 0.13 CmmolHA $CmmolS^{-1}$, respectively, were obtained.

Since the system seemed to be more specialized in GB accumulation rather than PHA, to utilize the available carbon sources more efficiently for PHA production, the introduction of a pre-fermentation step should be explored. The carbon compounds present in HSSL could be converted into SCOA through acidogenic fermentation. This strategy would provide a more suitable substrate for PHA production by MMC and, consequently, higher PHA contents could be expected.

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Chapter 5

Analysis of microbial community evolution when acclimatized to HSSL for PHA production

The key to optimizing PHA-production processes by mixed microbial cultures is the maximization of the selective pressure imposed on culture enrichment. Monitoring population evolution will allow for a better understanding of the relation between factors influencing microbial selection and the resultant community structure.

The microbial community of a HSSL-fed selection reactor operated under feast and famine conditions was identified and quantified through FISH, DGGE and 16S rRNA gene clone library. The microbial community was found to be extremely dynamic even during the pseudo-stationary state of the reactor operation as revealed by FISH and DGGE analysis. The community was dominated by *Alphaproteobacteria*, enclosing bacteria belonging to *Paracoccus* and *Rhodobacter* genus, both PHA-storing microorganisms.

The complexity of the raw material used as substrate allowed for the presence of different bacterial groups as side population, which consumed the different carbon sources without competition. *Agrobacterium*, *Flavobacteria* and *Brachymonas* were identified by 16SrRNA gene clone library, and never reported as PHA producers. Nevertheless, a significant number of bacteria able to accumulate PHA were detected in the enriched culture.

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5.1. Introduction

Plastics have gained importance as a way to enhance life quality and comfort and are present in our everyday life in all sorts of forms: from disposable utensils to packaging (Chen, 2009; Kamm et al., 2006). Their applications relay on properties such as strength, lightness, malleability, durability and resistance to degradation. However, these characteristics are now a major problem since they make their disposal a challenge (Kamm et al., 2006). Accumulation of microscopic plastic debris at sea is particularly alarming as well as the exponentially increasing need of landfill for municipal solid waste disposal (Gomez et al., 2012).

In recent years, bioplastics arose as an alternative to conventional ones to tackle all the disposal problems. PHA can be highlighted due to their total biodegradability and biocompatibility and the possibility of being produced from non-edible raw materials. PHA production by MMC and wastes or surplus-based feedstocks could be performed in a tree-step methodology. This starts with an acidogenic fermentation of organic components of the feedstock into SCOA, followed by a culture selection stage where the imposed operational parameters will allow selecting a robust and homogenous PHA-storing culture. The final step comprises the PHA accumulation design to maximize the amount of polymer stored by cells withdrawn from the previous step and fed with the substrate retrieved from the first one (Dionisi et al., 2004).

The selection step is crucial to develop a sustainable PHA production process since bacteria with no or low PHA accumulation capacity are removed from the reactor (Albuquerque et al., 2013; Queirós et al., 2015; Reis et al., 2011). The community analysis of the population is an important aspect, and together with tuning of operational parameters, could led to optimal processes. Efforts on assessing the PHA production potential and to comprehend which fraction of the starting culture is capable of accumulating PHA do not have the due prominence. These factors hinder a quick implementation of a possible industrial process based on MMC (Queirós et al., 2015).

Queirós et al. (2015) reviewed studies that applied molecular tools to elucidate the microbial diversity and monitor temporal and responsive community dynamics in PHA production process. Only few studies used wastes or surplus-based feedstocks to

acclimatize an MMC (Queirós et al., 2015). Using fermented sugar molasses, Albuquerque et al. (2010) were able to enrich an MMC where 88% of the microorganisms revealed PHA-storing capacity. This culture was dominated by *Azoarcus* and *Thauera* (88% of the total bacteria population), both belonging to *Betaproteobacteria* class. In accumulation tests, authors achieved 74.6% cdw of P(3HB-*co*-3HV) (Albuquerque et al., 2010). Moita and Lemos (2012) achieved a stable microbial community after 167 days of operation using bio-oil from fast-pyrolysis of chicken beds. Changes introduced during the operation process, SRT and mineral medium composition, led to a culture dominated by *Betaproteobacteria*, constituted mainly by *Thauera* and *Zooglea* and a small fraction of *Alphaproteobacteria*, mostly *Amaricoccus* (Moita and Lemos, 2012). All the above microorganisms are already reported to be involved in PHA accumulation. Jiang et al. (2012) using fermented paper mill wastewater and strict operational control: temperature and pH control at 30 °C and 7, respectively, and a HRT and SRT of two days, selected a culture constituted by 56% of *Plasticicumulans acidivorans*. The culture reached the highest amount of PHA, so far reported, with an MMC and real substrate, 77% cdw P(3HB-*co*-3HV) (Jiang et al., 2012).

HSSL was already tested as a possible substrate for PHA production by an MMC, which reached a content of 67.6% cdw (Queirós et al., 2014), with a microbial community dominated by *Alphaproteobacteria* (72.74%). Despite proving possible the PHA production, a steady-state was not reached. Later, Queirós et al. (2016) studied the long term operation with the same substrate, but with a higher amount of lignosulphonates (LS) (≈ 160 g L^{-1}) reaching the steady-state after 250 days of operation (Queirós et al., 2016). The objective of the present study was to evaluate the microbial community evolution during the operation of this last reactor and to infer how the parameters imposed and the fluctuations of the substrate composition influenced the microbial dynamics.

5.2. Materials and Methods

5.2.1. Reactor Operation

The reactor was operated as described by Queirós et al. (2016). A SBR was inoculated with activated sludge obtained from the aerobic tank of the municipal WWTP Aveiro Norte (SIMRia). The SBR was operated under ADF conditions, during which alternating feast and famine phases were imposed. The reactor was fed with HSSL, with an OLR of 17 g COD L^{-1} day^{-1}. The SBR was operated for 430 days (86 SRTs), in cycles of 8 h that comprised 7 h of aerobiosis, with fresh medium supplied during the first 0.25 h, 0.5 h of settling and 0.25 h of withdrawing. The HRT and SRT were 1 and 5 days, respectively. Regular samples were collected for different molecular analysis of the microbial community.

5.2.2. Fluorescence *in situ* Hybridization

FISH was performed on paraformaldehyde-fixed biomass samples (Amann et al., 1995). Aiming to follow the culture evolution, several oligonucleotide probes were applied. All the hybridizations with group specific probes (Annex A) were carried out simultaneously with probes EUB338, EUB338-II and EUB338-III combined in a mixture (EUB338mix) for the detection of most bacteria, and with DAPI staining for quantifying the total number of cells. All the probes were synthesized with FITC and Cy3 labels and purchased from MWG AG Biotech (Germany). With the goal of evaluating the PHA accumulating capacity of the culture, Nile blue staining was applied to fresh samples taken from the SBR at the end of the feast phase using the method of Ostle and Holt (Ostle and Holt, 1982). Both FISH and Nile blue samples were viewed using epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software).

5.2.3. Denaturing Gradient Gel Electrophoresis

The evolution of the bacterial community structure and composition during the SBR operation was also investigated via PCR-DGGE analysis of 16S rRNA genes. Metagenomic DNA was extracted from sludge pellet collected at each sampling time with

the UltraClean Microbial DNA kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The efficiency of extraction was determined by direct nanodrop measure.

From DNA extracted, 16S rDNA fragments were amplified with the primers shown in Table 5.1, which are specific for universally conserved bacterial 16S rDNA sequences. PCRs were performed with a mixture of 5x RANGER Reaction Buffer 10 µL, which contains dNTPs, $MgCl_2$ (1.5 mM final concentration) and enhancers; 50 pmol of each primer, 5 µL of DNA template and water up to 50 µL. The protocol was developed for a standard 50 µL amplification of 10 kb fragments. The PCR reaction was programed to do an initial denaturation at 95 °C for 1 minute and then repeat for 35 cycles the following steps: 10 seconds denaturing step at 95 °C; 30 seconds annealing step at 58 °C; 1 min extension at 68 °C. Finally, an extension step of 7 minutes at 68 °C was performed. The samples were cooled down until 4 °C and a 2% (w/v) agarose gel electrophoresis was performed to check if there was amplification.

PCR products were loaded onto an 8% (w/v) polyacrylamide gel in ½ x TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA at pH 8). Initially the 8% (w/v) polyacrylamide gels were made with a denaturing gradient ranging from 20 – 80% and then the gradient was narrowed to 45 – 55%. Denaturant (100%) contained 7 M urea and 40% formamide. The electrophoresis was run at 60 °C, for 10 minutes at 20 V, and subsequently for 16 hours at 60 V. After electrophoresis, gels were stained for 30 minutes with SYBR Safe DNA gel stain and then observed and photographed in Safe Imager 2.0 Blue-Light Transilluminator.

Digitized DGGE images were analyzed with Bionumerics software. Lanes and reference bands were applied to each gel image by the software, with manual fine-tuning of the band designations. Subsequently, similarities between the densitometric curves of the band patterns were calculated based on the Pearson coefficient and DGGE patterns were clustered based on the unweighted pair group method with arithmetic mean (UPGMA) clustering algorithm. PCA and evenness and diversity coefficients were equally calculated resorting to Bionumerics software.

5.2.4. Genomic DNA extraction and PCR amplification of 16S rRNA gene

DNA was extracted from 2 mL of the MMC sample following the protocol reported in Rossetti et al. (2003). The concentration and purity of the genomic DNA were determined by NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). 16S rRNA genes of genomic DNA were amplified using primers 27f and 1492r for Bacteria domain using TaKaRa Ex TaqTM kit (Japan) as previously described (Rossetti et al., 2003). T7f and U19r (or M13r), which are specific plasmid primers, were used for the screening of clones from the 16S rDNA clone library (Table 5.1). PCR products were purified using the QIAquick® PCR purification kit (Quiagen, Milan, Italy). 16S rRNA gene sequences of the clone inserts were obtained using the following primers: 530f, 926f, 907r and 519r (Table 5.1).

Table 5.1. Primers for DGGE V6 to V8 Amplification and PCR and sequencing for clonal analysis.

	Primers	Sequence (5' – 3')
PCR	27f	AGAGTTTGATCMTGGCTCAG
	1492r	TACGGYTACCTTGTTACGACTT
	T7f	TAATACGACTCACTATAGGG
	U19r	GTTTTCCCAGTCACGACGT
	M13r	TCACACAGGAAACAGCTATGAC
Sequencing	530f	GTGCCAGCMGCCGCCG
	926f	AAACTYAAAKGAATTGACGG
	907r	CCGTCAATTCMTTTRAGTTT
	519r	GWATTACCGCGGCKGCTG
DGGE	GC 968f	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGG GGGAACGCGAAGAACCTTAC
	UNI 1401R	CGGTGTGTACAAGACCC

M = C:A; Y = C:T; K = G:T; R = A:G; W = A:T; all 1:1

5.2.5. Cloning of 16S rRNA gene

Cloning of PCR products was carried out using pGEM-T Easy Vector System (Promega, USA) into *Escherichia coli* JM109 competent cells (Promega, USA) according to the manufacturer's instructions. Positive inserts were amplified from recombinant plasmids obtained from white colonies by PCR using the sequencing primers T7f and M13r following the PCR protocol previously described.

The nucleotide sequences identified in this study were deposited in the GenBank database under accessions numbers KR081266, KR081267, KR081268, KR081269, KR081270, KR081271, KR081272, KR081273, KR081274, KR081275.

5.3. Results and Discussion

The MMC characterized in this study was selected by inoculating a SBR with activated sludge, obtained from the aerobic tank of the municipal WWTP Aveiro Norte (SIMRia), and imposing aerobic dynamic feeding (ADF) conditions. HSSL was used as substrate, with an organic loading rate (OLR) of 17 gCOD L^{-1} d^{-1} and 1 and 5 days of hydraulic retention time (HRT) and SRT, respectively. The SBR was operated for 430 days (86 SRTs) (Queirós et al., 2016).

The reactor reached a pseudo-steady state (PSS) by day 260 with a feast/famine (F/F) ratio of 0.25 and dropping to 0.125 by day 331 of operation. These values are, usually, indicative of a good PHA storage response (Reis et al., 2011) and the change, from 0.25 to 0.125, indicates that the culture was still being selected according to the parameters imposed (Fig. 5.1). Hence, a microbial characterization is fundamental to understand where and how the imposed operational parameters could be tuned. Despite the F/F ratios obtained, the highest PHA content obtained during the selection process was 6.6% on day 237 but the highest PHA storage yield occurred on day 270, 0.49 Cmmol HA Cmmol S^{-1}. The PHA content was slightly lower than the range, 9 – 10%, observed for the selection step in other reported works (Albuquerque et al., 2007; Moita and Lemos, 2012). The extent of PHA storage estimated microscopically at cell level by Nile Blue staining was in line with this finding (data not shown). Furthermore, the results obtained from accumulation assays performed, around 7% PHA cdw, were far from those reported in the literature 47% - 77% PHA cdw (Jiang et al., 2012; Moita et al., 2014; Queirós et al., 2016). Besides PHA, a higher glucose biopolymer storage yield was obtained, 18%. This might be due to the complexity of the raw material used, allowing different bacterial groups to persevere in the culture, which consumed the different carbon sources without competition (Queirós et al., 2016).

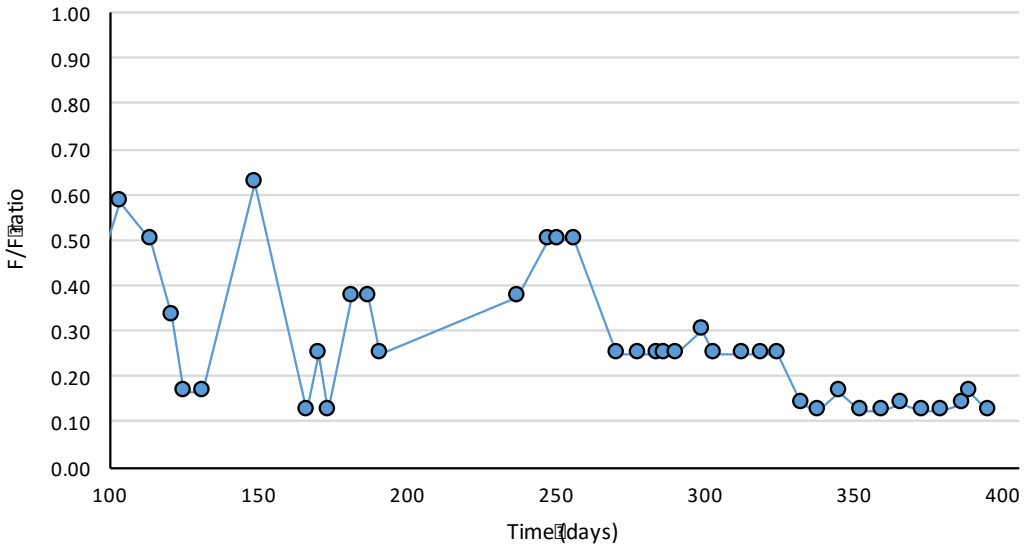

Fig. 5.1. Feast-to-Famine ratio along the reactor operation.

5.3.1. Microbial Community Analysis

With the purpose of understanding the microbial processes taking place in the bioreactor, the study of the microbial community and its evolution is required. As such, the bacterial community selected in the SBR was characterized through the operational time by fluorescence *in situ* hybridization (FISH) analysis. After this approach, the microbial evolution process occurring in the SBR was investigated resorting to a culture independent molecular method, denaturing gradient gel electrophoresis (DGGE) and to a 16S RNA gene clone library.

The adopted strategy for community analysis along the reactor operational period through FISH technique started at the higher taxonomic levels testing the culture with specific probes for the *Archaea* and *Bacteria* domains. The screening for the presence of *Archaea* did not show hybridization in any of the analyzed samples while Bacteria was abundantly present as seen in Fig. 5.2. Next, a successively refinement of the taxonomic composition belonging to *Bacteria* domain until the genus level was performed. The result of this analysis allowed to monitor the evolution of the identified taxonomical bacterial groups in the microbial community throughout the entire reactor operation.

5.3.2. FISH analysis

The original activated sludge community was quite diverse, being detected Bacteria belonging to *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Chloroflexi*, *Flavobacteria*, *Bacteroides* and *Firmicutes*. They accounted for 48 - 93% of EUBmix hybridization cells, meaning that during some periods other classes not targeted were also present.

Observing the data, the most abundant class of organisms since 270th day of operation was *Alphaproteobacteria* that increased from 38% to 65%. This dominance roughly coincides with the first stabilization of the F/F ratio. The next most representative class was *Betaproteobacteria.* This class shared a co-dominance over the culture until day 187, remaining stable until 287th day of operation. The percentage of hybridization of specific probe for the group of classes *Flavobacteria*, *Bacteroides* and *Sphingobacteria* revealed some fluctuations during the whole operation period. Regarding *Gammaproteobacteria*, its content remained constant around 7 - 10% of the total EUBmix hybridization. Several PHA accumulating microbial communities characterized in the literature reported *Betaproteobacteria* as the major class present (Albuquerque et al., 2013; Jiang et al., 2011; Moita and Lemos, 2012). However, using the same feedstock, Queirós et al (2014) described a similar community composition although *Deltaproteobacteria* and *Actinobacteria* were also found to be present (Queirós et al., 2014). The *Alphaproteobacteria* progressive increase followed the acetic acid uptake rate increase, one of the main precursors for PHA accumulation thus being possibly related (data not shown). However, correlation between microbial classes composition and PHA accumulation yield or PHA content does not appear to be evident.

Operational parameters imposed shaped the microbial community diversity and relative abundance. Queirós et al. (2014) used the same by-product to acclimatize an MMC, but the different imposed conditions in the present work such higher OLR and shorter cycle lead to a complete different culture and more dynamic given the results obtained by FISH and DGGE.

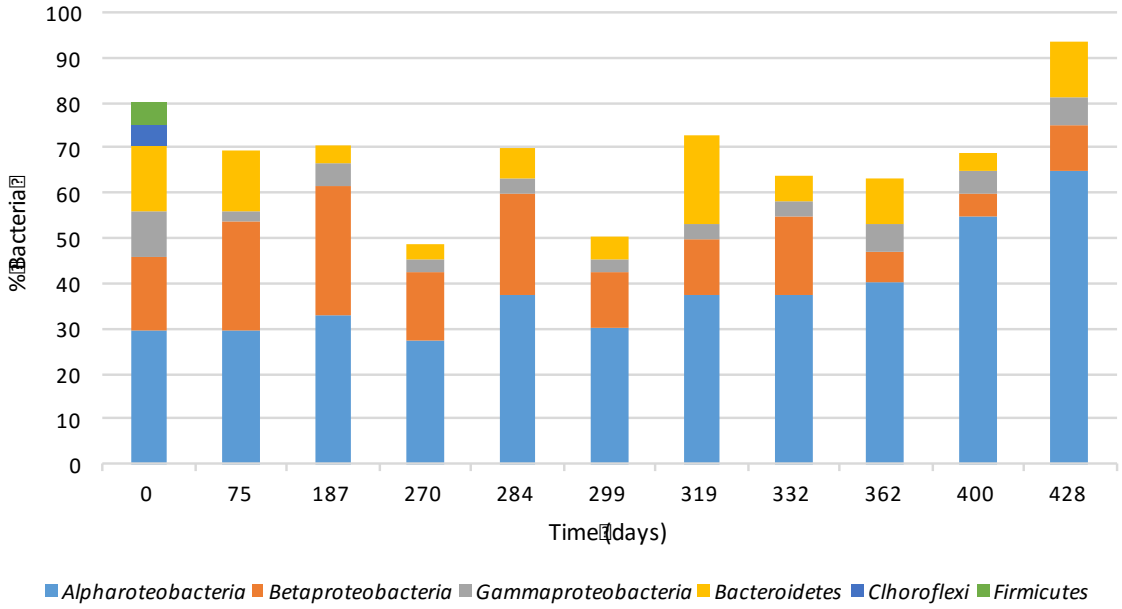

Fig. 5.2. Microbial community evolution analyzed through FISH.

The *Alphaproteobacteria,* and *Betaproteobacteria* classes, as the most representative of the microbial culture, were further studied at genus level. From the several probes tested, which are specific for several genera usually reported as PHA-accumulating microorganisms, only two genera of *Alphaproteobacteria* were found: *Paracoccus* and *Rhodobacter,* Fig. 5.3. Between these two genera only *Paracoccus* was identified by Queirós et al (2014). However, several studies reported *Rhodobacter* as being present in selected PHA-accumulating cultures and this genus is known to be able to produce PHA (Ciesielski et al., 2010; Yang et al., 2012). Despite the clear dominance by *Alphaproteobacteria* class, these two genera suffered significant fluctuations over the operation period, not following the class evolution.

It should be noted that several specific probes that hybridize with known PHA accumulating organisms (*Thauera, Amaricocus, Azoarcus* and *Zooglea* genus) were also tested with negative results obtained. The microscopic images with positive result for the probes tested in sample of 428th day of operation are presented in Fig. 5.4. All the other probes tested were negative.

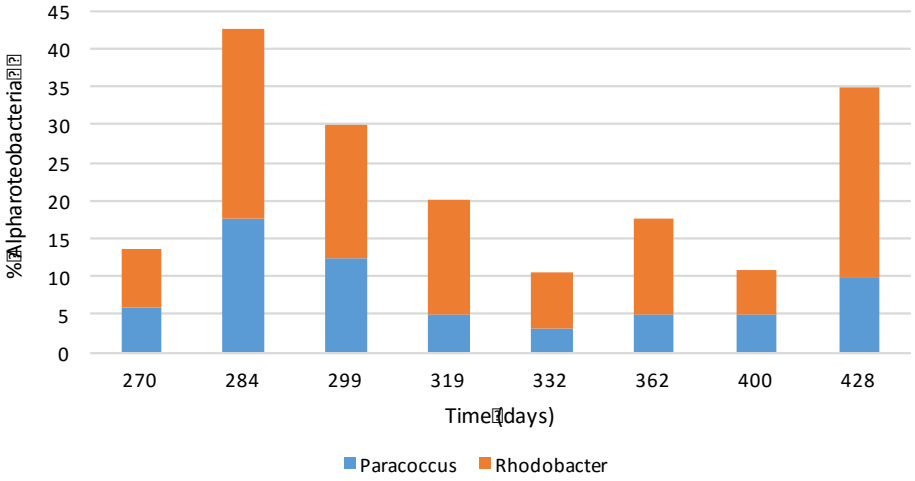

Fig. 5.3. Partial *Alphaproteobacteria* community constitution identified by FISH.

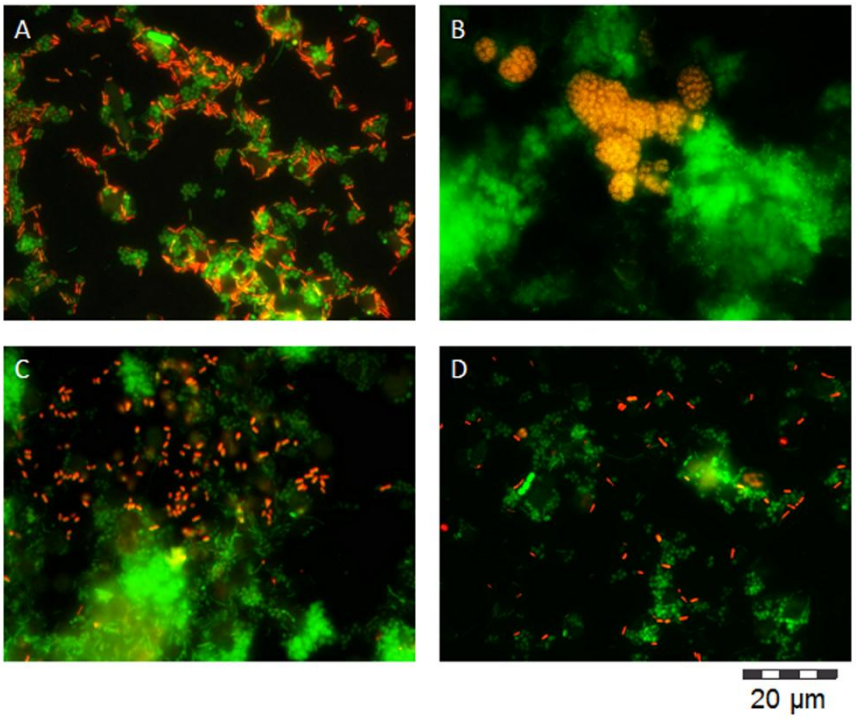

Fig. 5.4. FISH pictures from the last sample, day 428: A – *Alphaproteobacteria*, B – *Betaproteobacteria*, C – *Paracoccus*, D – *Rhodobacter.*

Cells that hybridized with BET42a probe were always presented in cocci aggregates as seen in Fig. 5.4 B. It would be interesting to establish a link between this singular morphology and the identity of the genus to accelerate future analysis of this communities. Cell-sorting reverse transcription PCR could be further applied to identified

in particular these bacteria (Lemos et al., 2008). At the genus level, only *Paracoccus* and *Rhodobacter* belonging to *Alphaproteobacteria* had positive results. In both cases, Fig. 5.4 C-D, a coccobacilli morphology was verified.

5.3.3. DGGE analysis

To correlate the operational conditions with the bacterial richness and dynamics in different stages of the process, PCR-DGGE technique was applied. As 16S rRNA-based fingerprinting is useful for describing dynamics in microbial community structure rather than diversity, DGGE was used to run samples from the PSS state to try to validate the variability observed by FISH. A few samples were selected and the DGGE profiles obtained for each sampling day are shown in Fig. 5.5.

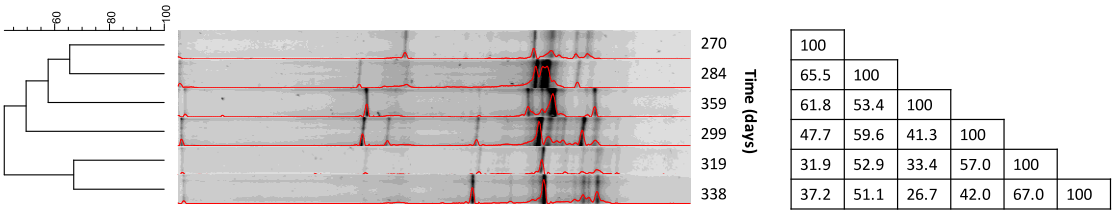

Fig. 5.5. DGGE profile of the bacterial community structure throughout PSS and respective cluster analysis tree constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering algorithm. Similarities were calculated using Pearson's coefficient, using BioNumerics software.

DGGE fingerprints revealed the microbial changes during the SBR operation period with some shared bands in all samples. Although, and taking into consideration that the analysis was done during the PSS, some bands were present in a single lane contrary to what was described in some works (Dionisi et al., 2005; Valentino et al., 2015). Interestingly, Valentino et al (2014) observed that, despite the highest variability in microbial composition occurred only in the first weeks, some of phylotypes were dominant only for a certain amount of time (Valentino et al., 2014). This suggests that a succession of different PHA producers occurred during the long operation period of the SBR.

Cluster analysis was performed using the Pearson's coefficient. The dendogram showed significant variations in bacterial communities during the PSS. The largest shift in

bacterial assemblage was identified between samples from 338 to 359 day, the same period when F/F decreased from 0.25 to 0.125. The similarity of the bacterial community during the first PSS (F/F = 0.25) varied from 65.5% and 31.9%, indicating a large shift of population occurring during a stable period of operation. Despite the high variability observed during the PSS no significant differences on stoichiometric and kinetic parameters of the referred days were observed. These results confirm, on a molecular base, the ineffectiveness of imposed conditions in progressively selecting a predominant restricted microbial population from the initial inoculum. Therefore, a specialized PHA storage community was not achieved, confirming the poor performance of the reactor

The complexity of the raw material used as substrate probably allowed different bacterial groups to be present in the culture, which consumed the different carbon sources without competition and at different rates (Jiang et al., 2012; Marang et al., 2014; Moita and Lemos, 2012). Although analysis of daily cycles demonstrated an MMC preference for substrate storage as reserve polymers, substantial enrichment of a PHA accumulating culture was not found in comparison with others works in the (Albuquerque et al., 2007; Jiang et al., 2012, 2009). The more easily biodegradable fraction of carbon present in the HSSL was consumed by PHA and GB accumulating organisms. Populations without the ability to store polymers could grow and persisted in the system throughout the consumption of the less biodegradable carbon fraction. Hence, since the PHA storage capacity of the system was maintained constant during the two PSS (Queirós et al., 2016), the high shift of population verified in this period of time could be explained by shifts on the non-PHA storing organism present in the microbial consortium.

To better visualize the relationships among samples, a binary matrix was constructed based on the presence or absence of bands. The resulting matrix was used to conduct a principal component analysis (PCA) analysis. In PCA analysis, PC1 captured 37.5% of variance and PC2 captured 31.5%, totalizing 69.0% of variance. The subsequent PCs captured progressively lower variance percentages, thus only the loadings of the first 2 principal components were analyzed.

In agreement with results obtained during cluster analysis of the DGGE fingerprinting, the PCA analysis showed a lack of relation between all samples (Fig. 5.6).

At most, two clusters would be expected representing the two PSS observed. This demonstrated the huge variability on microbial population despite the operating conditions remaining the same.

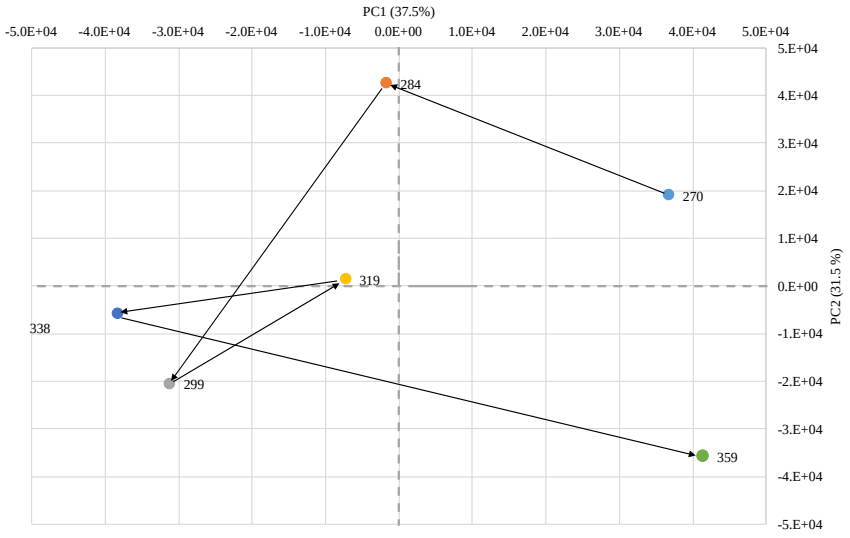

Fig. 5.6. PCA analysis using the presence/absence matrix of the DGGE profiles, resorting to BioNumerics software. PC1 and PC2 captured 69% of variance (37.5% and 31.5%, respectively).

Shannon's and evenness's indexes were calculated (Table 5.2). No significant differences could be observed between samples when the Shannon diversity index was considered, being all the values on the low range. The community evenness index of all samples was close to one and no significant differences were obtained between samples. These results indicated that the distribution of microbial groups during both PSS were uneven, probably due to the high diversity of carbon sources present in the HSSL which could be metabolized by different microbial populations (PHA storing and non-PHA storing organisms).

An inherent expectation is that during PHA-storing microorganisms' enrichment there would be a tendency for the microbial richness to decrease over time with a relative sustained stability (Morgan-Sagastume et al., 2010; Queirós et al., 2015; Valentino et al., 2015, 2014). However, some studies already observed diverse microbial communities' composition under constant changes in relative abundances of populations under PSS conditions of reactor performance (Janarthanan et al., 2016; Liu et al., 2013;

Valentino et al., 2014). In fact, Liu et al. (2011) observed an increase in the number of DGGE bands from the acclimated sludge samples in comparison to the non-acclimatized sludge sample (Liu et al., 2011). Such results showed that significant variability of the storage responses could not be not only dependent on changes in the microbial community but also due to the biomass physiological state (Queirós et al., 2015) .

Table 5.2. Shannon's diversity index and evenness index of each sample analyzed trough DGGE, calculated with BioNumerics software.

Operation day	Shannon Diversity Index	Eveness Index
270	1.8928	0.9134
284	1.8187	0.8607
299	2.1152	0.8459
319	2.0148	0.7375
338	1.8531	0.8487
359	1.8209	0.8543

5.3.4. 16S rRNA gene Clonal Analysis

Although FISH provided the overall structure of the microbial community at group level, a more detailed information on the main bacterial components is necessary to explain the rather low PHA accumulations when good F/F ratio was achieved and rather stable stoichiometric values obtained. 16S RNA gene clone library was constructed to better understand such results, Fig. 5.7. Although the results were in accordance to FISH analysis, large number of clones with sequences belonging to *Alphaproteobacteria* were amplified, which were representative of bacteria that do not accumulate PHA. Bacteria such as *Agrobacterium*, *Flavobacteria* and *Brachymonas* were detected and never reported as PHA-storing organisms but able to consume the sugar fraction. Ferreira et al. (2016) were able to isolate microorganisms from a culture acclimatize to HSSL which were able to consume xylose and store PHA (Ferreira et al., 2016). Despite the existence of bacteria able to convert xylose and other carbohydrates into PHA, probably their presence in the selected MMC was residual and the compounds were used to produce glucose polymer. A considerable number of species highlighted by the clonal analysis

were reported as PHA-accumulating bacteria and previously identified or isolated from activated sludge samples, such as *Bacilus* spp. (Emeruwa and Hawirko, 1973), *Rhodobacter* spp. (Jiang et al., 2009), *Flavobacterium* spp. (Majone et al., 2006), *Leadbetterella* spp. (Shen et al., 2015; Valentino et al., 2014) and *Ectothiorodospira* spp (Quillaguamán et al., 2010; Villanueva et al., 2010). These results showed that despite the diversity of microorganisms selected, almost all of them had the capacity to accumulate PHA. So, the lower accumulation probably occurred due to the biomass physiological state instead of being related to a poor selection, as previously inferred by DGGE analysis.

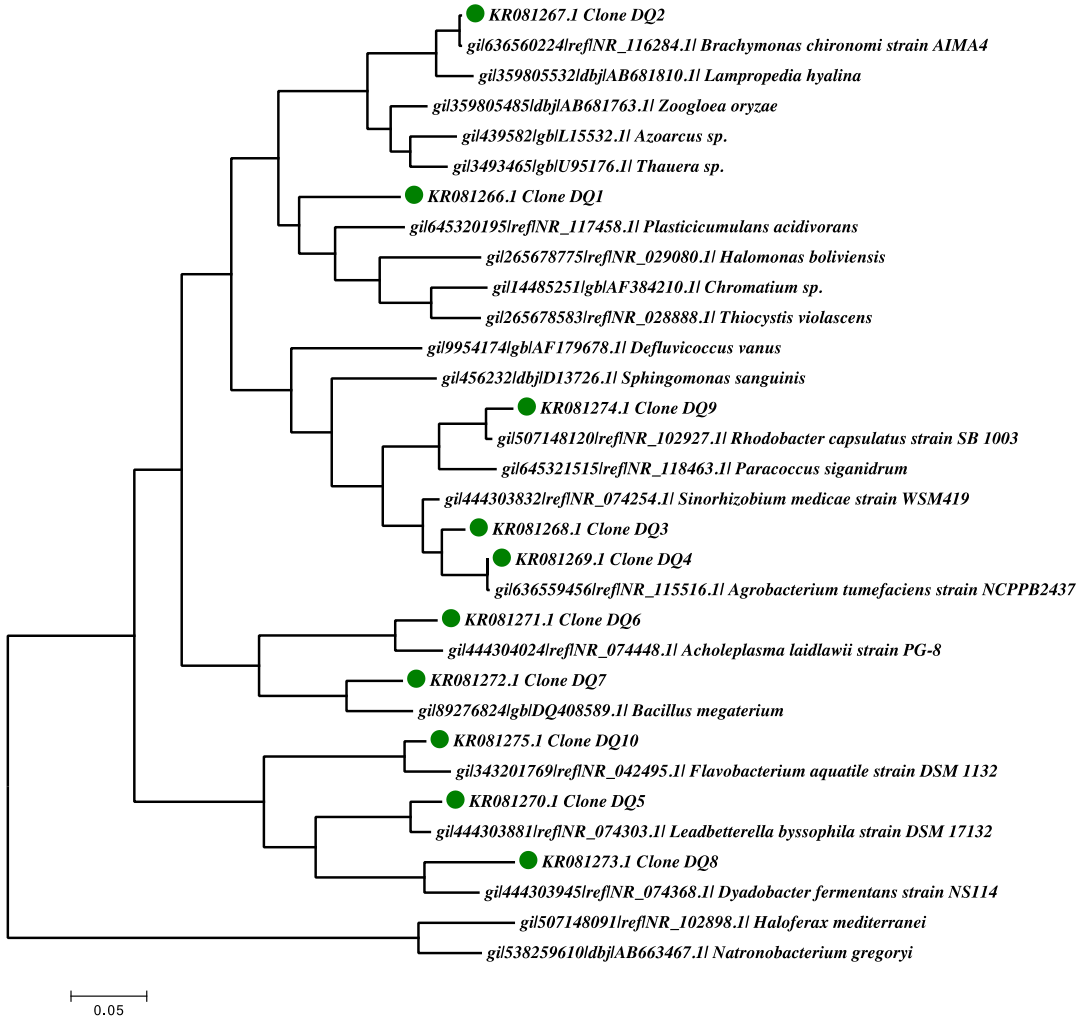

Fig. 5.7. Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Evolutionary analyses were conducted in MEGA6. Clones DQ1 – DQ10 are preceded by a green filled dot.

5.4. Conclusion

Considering the microbial characterization, new approaches should be planned to achieve a better selection in PHA-storing microorganisms. Contrary to what was observed by Queirós et al. (2014), Queirós et al. (2016), with shorter cycle lengths and higher OLR, were unable to select a culture capable of high PHA accumulation. MMC are known to preferentially consume SCOA and to not use sugars for PHA production (Carta et al., 2001; Dircks et al., 2001; Moita and Lemos, 2012) Sugars consumption is associated with glycogen storage since it is more energetically favorable than PHA storage, as observed by Queiros et al. (2016). Hereupon, a new strategy could be based on the acifidication of HSSL to remove the sugar fraction and some toxic components converting them into SCOA. With this, it is expected to decrease, or even eliminate, the glucose polymer content and shorten the adaptation period, getting a more specialized culture.

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Chapter 6

Impact of organic acids supplementation to HSSL as substrate for selection of PHA-producing organisms

The effectiveness of PHA production process from a waste stream is determined by the selection of a suitable MMC. In this work, a feedstock of the paper industry, HSSL, supplemented with SCOA to simulate a fermented effluent, was used as substrate to enrich an MMC in PHA-storing microorganisms. A pseudo-stationary state was reached after 34 days of SBR operation, using an SRT of 5 days and a 24 h cycle later changed to a 12 h cycle, the last with a higher OLR. In the accumulation step, the ability of the selected MMC to accumulate PHA was tested. The selected MMC reached a maximum PHA content of 34.6% (3HB:3HV – 76:24). The bacterial community was analyzed through FISH analysis. Bacteria belonging to the four main classes were identified: *Betaproteobacteria* (44.7 ± 2.7%), *Alphaproteobacteria* (13.6 ± 1.3%) and *Gammaproteobacteria* (2.40 ± 1.1%) and *Bacteroidetes* (9.20 ± 3.8%). Inside the *Betaproteobacteria* class the dominant genus was found to be *Acidovorax* (71%). A clone library was prepared and several of the identified clones were already described as PHA-producing organisms. The selection strategy through the manipulation of cycle length and OLR led to an MMC enriched in PHA-storing organisms despite the complexity of the feedstock.

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Queirós, D., Rangel, C.., Lemos, P. C., Rossetti, S., Serafim, L. S.. Impact of organic acids supplementation to hardwood spent sulfite liquor as substrate for selection of polyhydroxyalkanoates-producing organisms.

6.1. Introduction

The Confederation of European Paper Industries (CEPI) on its "Preliminary Statistics 2015 Paper and board production" state that CEPI member countries' production fell slightly, by around 0.3% in 2015 after -0.2% in 2014 (CEPI, 2016). In Portugal, the tendency was contrary, with exportations increasing and investments being still made. The Navigator Company invested 159 million euros to dominate the European market in tissue paper production and ALTRI group bet in the manufacture of soluble paste - used in the textile industry for the manufacture of viscose, with increasing demand from China. This represents a massive side production of wastes and residues that could be valorized in a lignocellulosic-based biorefinery (Fava et al., 2015; Koutinas et al., 2014).

Part of pulp and paper industry's residues are usually burnt for energy production and the lignin and lignosulphonates are already used as oil well drilling additives, concrete additives, dyestuff dispersants, agricultural chemicals, animal feed and other industrial binders (Norgren and Edlund, 2014). However, a significant fraction of these residues, namely SSL, can be converted into value-added products. SSL, a by-product of acidic sulfite wood pulping process, are rich in C5 and C6 sugars that can be used for the production of succinic acids (Alexandri et al., 2016), SCOA, and biofuels such as bioethanol (Pereira et al., 2012). SSL also contains sugar hydrolysis products such as acetic acid that can be used for PHA production (Queirós et al., 2016, 2014).

The environmental hazards brought by conventional plastics represent a major concern to today's Society. Such problem can be tackled through the production of biodegradable plastics as PHA, especially, if by-products, such HSSL, and MMC can be applied (Serafim et al., 2008). Such strategy allows for a cost reduction, since PHA industrial production, so far, relies on pure culture and high-cost synthetic substrates (Serafim et al., 2008). In the last 16 years, an effort is being made to develop cheaper alternatives for PHA production processes based on the use of industrial wastes and by-products as substrate (Albuquerque et al., 2010; Dionisi et al., 2004; Jiang et al., 2012; Moita et al., 2014; Queirós et al., 2016). However, researchers are still struggling with the lower PHA content and lower volumetric productivities achieved by MMC when

compared with the pure cultures. To overcome such problem, a three-step process was designed (Dionisi et al., 2004). The bottleneck of this process is the selection of a stable MMC with a high PHA storage capacity (Queirós et al., 2015). This can be achieved by subjecting microbial cultures to alternate periods of short carbon availability ("feast") followed by a prolonged unavailability ("famine"), called as aerobic dynamic feeding (ADF). The resulting selective pressure can be imposed by manipulating several parameters such as OLR and cycle length, taking into consideration that a balance between growth and accumulation should be attained. Higher OLR increases the volumetric productivity of mixed culture PHA production processes. It could be expected that the larger the quantity of carbon supplied, the higher the cellular concentration will be (Albuquerque et al., 2010; Dionisi et al., 2006; Valentino et al., 2015). The shortening of cycle lengths leads to higher numbers of SBR's cycles per day that consequently increases the influent flow rate (HRT being kept constant). Under those conditions the specific substrate uptake rate should be held constant. Higher biomass concentration and similar specific rates will result in superior volumetric rate without alteration on the F/F ratio (Reis et al., 2011, Albuquerquer et al., 2010). By applying ADF conditions, Johnnson et al. (2009) selected an MMC that reached a PHA content of 89% using acetic acid as substrate and, later, Jiang et al. (2012) obtained a PHA content of 77% cdw from fermented paper mill wastewater.

SCOA are the main precursors for PHA production by MMC. It was reported the incapability of MMC to store PHA from sugar-based compounds when submitted to feast and famine conditions (Carta et al., 2001). Queirós et al. (2016, 2014) observed that notwithstanding being able to select an MCC with HSSL under ADF conditions, presenting feast-to-famine ratios indicative of PHA storage, low accumulations values were obtained. Moreover, Queirós et al. (2016) observed the accumulation of a glucose polymer that probably was produced by a side population able to uptake sugars. In recent years, several studies dedicated to investigate MMC structure in PHA producing processes, revealed a broad phylogenetic diversity, as reviewed by Queirós et al., (2015). Still, a link between operational conditions and the growth of one specific organism over the others,

as well as the impact of PHA-accumulating organisms on the overall PHA accumulation, remains to be established (Albuquerque et al., 2013; Carvalho et al., 2014).

The objectives of this work were: to investigate the feasibility of enriching an MMC in PHA-storing microorganisms with HSSL supplemented with SCOA (acetic, propionic and butyric acids) and to follow the microbial community evolution through biological molecular analysis. The goal of the supplementation was to decrease the adaptation period and infer if a pre-fermentation step would be justified. Moreover, the effect of manipulating the OLR and cycle length on the MMC selection was also evaluated.

6.2. Material and Methods

6.2.1. Culture medium

HSSL from magnesium-based acidic sulfite pulping of *Eucalyptus globulus* was supplied by Caima – Indústria de Celulose S.A. (Constância, Portugal). Pre-evaporated HSSL was collected from an inlet evaporator of a set of multiple-effect evaporators to avoid the presence of free SO_2. To remove part of the most recalcitrant compounds, HSSL was submitted to a preliminary pretreatment (Queirós et al., 2014). The pre-treatment started with a pH adjustment to 7.0 with 6 M KOH, followed by aeration with compressed air (6 h per L of HSSL). Then, the suspension was centrifuged for 1 h at 5000 rpm. The precipitated colloids were filtered off using a 1 μm glass microfiber filter. Finally, the total COD of pretreated HSSL was determined (205 gCOD L^{-1}). LS were still the main constituents (120 – 160 g L^{-1}) along with xylose and acetic acid (50 and 18 g L^{-1}, respectively). No phosphates and ammonia were detected in HSSL.

HSSL was diluted with a mineral solution (1:45). The mineral solution was composed by (per liter of distilled water): 160 mg $MgSO_4 \cdot 7H_2O$, 80 mg $CaSO_4 \cdot 2H_2O$, 20 mg $FeCl_3$, 8 mg $Na_2MoO_4 \cdot 2H_2O$, 160 mg NH_4Cl. The medium, after dilution, was supplemented with acetic, propionic and butyric acids, to obtain a final concentration of acids of 30 Cmmol L^{-1} comprising 12 Cmmol L^{-1} of acetic acid, 10 Cmmol L^{-1} of butyric acid and 8 Cmmol L^{-1} of propionic acid. The pH of the medium was adjusted to 7.0 and the medium was autoclaved for 20 min at 121 °C. Under sterile conditions, KH_2PO_4 (16 mg L^{-1})

and K_2HPO_4 (64 mg L^{-1}) were added to the medium. Thiourea (400 mg L^{-1}) was also added to inhibit nitrification.

6.2.2. Reactor operation

The SBR was inoculated with activated sludge obtained from the aerobic tank of the municipal WWTP Aveiro Norte (SIMRia). The SBR working volume was 1.5 L and it was operated under ADF conditions, during which alternating feast and famine (F/F) phases were imposed.

Initially, the SBR worked in cycles of 24 h that comprised 22.5 h of aerobiosis, with fresh medium supplied during the first 15 min, 1 h of settling (with agitation and aeration switched off) and 0.5 h of withdrawing, resulting in a hydraulic retention time (HRT) of 2 days. An SRT of 5 days was imposed by purging 300 mL at the end of the aerobic period. The cycle duration was changed to 12 h comprising 10.5 h of aerobiosis, 1 h of settling and 0.5 h of withdrawing. The HRT and SRT were kept at 1 and 5 days, respectively. The OLR was 2.5 gCOD L^{-1} d^{-1} for 24 h cycles and 5 gCOD L^{-1} d^{-1} for 12 h cycles.

Reactor stirring (400 rpm), aeration and feeding and withdrawing pumps were controlled with timers. Dissolved oxygen and temperature were measured with Oxygen Meter Transmitter M300 (Mettler-Toledo Thornton, Inc). The system worked without pH and temperature control, although their values were monitored. To prevent foam formation, diluted silicone anti-foam (1:20) was manually added when excessive foam was observed. The SBR was cleaned on a daily-basis to prevent excessive biofilm formation on reactor walls and electrode surfaces.

SBR cycles were monitored periodically by taking samples across the entire reaction period. Samples were centrifuged at 14000 rpm and both supernatant and solid phase were stored for latter acetic, propionic and butyric acids, LS, ammonium, COD and PHA content quantifications.

6.2.3. Accumulation tests

Fed-batch tests (no nutrient limitation, with ammonium limitation, or with phosphorus limitation) were carried out in a bioreactor (BIOSTAT® A plus) without pH and

temperature control or sterile conditions. A respirometer was coupled to the bioreactor and recirculation of the medium was performed by a peristaltic pump. The stirring in the reactor and the respirometer was kept constant at 250 rpm, and the supply of air to the bioreactor was performed by an air pump (Boyu Air Pump, 8 L min^{-1}).

In each test, 1 L of biomass was collected from the main bioreactor, after withdrawn and before the feeding period, and inoculated to the fed-batch reactor. Five pulses of feed were supplied (each of 500 mL) to a final working volume of 3.5 L. Sampling was done every 15 minutes and, in parallel, the decrease on the concentration of dissolved oxygen was recorded for 3 minutes to determine the oxygen uptake rate.

6.2.4. Analytical methods

Biomass concentration was determined using TSS and VSS procedures described in *Standard Methods* (Clesceri et al., 1998).

PHA quantification and monomeric composition were determined using gas chromatography following the procedure described by Lemos et al. (2006). Glucose biopolymer (GB) was measured according to Moita et al. (2014).

COD was measured according to *Standard Methods* (Clesceri et al., 1998). Acetic, propionic and butyric acids were measured by HPLC following the procedure described by Queirós et al. (2014). LS was measured according to Restolho et al. (2009). LS concentration was calculated using the Beer-Lambert law with a molar attenuation coefficient of 7.41 g^{-1} cm^{-1} (Xavier et al., 2010).

Ammonium concentration was followed using a Crison Ion Selective Electrode after adding 100 µL of 0.9 M $Al_2(SO_4)_3$ solution to 1 mL of sample and applying a calibration curve obtained with standard solutions of NH_4Cl.

6.2.5. Microbial Community Analysis

6.2.5.1. Fluorescence *in situ* Hybridization

FISH was performed on paraformaldehyde-fixed biomass samples (Amann et al., 1995). All the hybridizations with group specific probes (Annex A) were carried out simultaneously with probes EUB338, EUB338-II and EUB338-III combined in a mixture

(EUB338mix) for the detection of most bacteria and with DAPI staining for quantifying the total number of cells. All probes were synthesized with FITC and Cy3 labels and purchased from MWG AG Biotech (Germany). With the goal of evaluating the PHA accumulating capacity of the culture, Nile blue staining was applied to fresh samples taken from the SBR at the end of the feast phase using the method of Ostle and Holt (1982). Both FISH and Nile blue samples were viewed using an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software).

6.2.5.2. 16S rDNA Clone Library

DNA was extracted from an SBR sample collected during the pseudo-stationary phase of operation (on 66th day, 12 h cycle). DNA extraction was performed using PowerSoil® DNA Isolation Kit following the protocol described by the manufacturer. The primers used to amplify 16S rDNA were 27 forward (f) and 1492 reverse (r), Table 6.1. The following PCR cycles were used: 98 °C for 2 minutes, followed by 35 cycles of 98 °C for 0.5 minutes, 58 °C for 0.5 minutes and 72 °C for 1 minute and, finally, an extension at 72 °C for 10 minutes. After the last cycle, samples were cooled down to 4 °C and an agarose (1%) electrophoresis was performed to check if there was amplification. Afterward, PCR products were purified using the QIAquick® PCR purification kit and quantified using the NanoDrop2000 Spectrophotometer.

Table 6.1. Primers used in PCR and sequencing.

	Primers	**Sequence (5' – 3')**
PCR	27f	AGAGTTTGATCMTGGCTCAG
	1492r	TACGGYTACCTTGTTACGACTT
	T7f	TAATACGACTCACTATAGGG
	U19r	GTTTTCCCAGTCACGACGT
	M13r	TCACACAGGAAACAGCTATGAC
Sequencing	530f	GTGCCAGCMGCCGCCG
	926f	AAACTYAAAKGAATTGACGG
	907r	CCGTCAATTCMTTTRAGTTT
	519r	GWATTACCGCGGCKGCTG

M = C:A; Y = C:T; K = G:T; R = A:G; W = A:T; all 1:1

The amplified 16S rDNA was ligated into pGEM®-T vector (Promega, USA) and transformed into *E. coli* JM109 Competent Cells (Promega, USA), per the manufacturer's instructions. The transformed cells were plated in LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37 °C. Clones were screened for insertion of the correct size by PCR amplification using primers T7f and M13r. The following PCR cycles were used: 96 °C for 10 minutes and PCR amplification was performed as already above. DNA sequencing was carried out by BioFab (Rome, Italy) using the primers 530f, 926f and 907r, Table 6.1. The complete sequences obtained were deposited in GenBank (accession numbers: KT262954, KT262955, KT262951, KT262956, KT262952, KT262957, KT262958, KT262959, KT262960, KT262961, KT262962, KT262953, KT262963, KT262964, KT2629).

6.2.6. Calculations

PHA content was calculated as a percentage of TSS on a mass basis:

$$\%\ \mathrm{PHA} = \frac{\mathrm{gHA}}{\mathrm{gTSS}} \times\ 100 \qquad (6.1.)$$

Active biomass (X) was obtained by subtracting PHA from VSS as (in g L^{-1}):

$$\mathrm{X} = \mathrm{VSS} - \mathrm{PHA} \qquad (6.2)$$

F/F was calculated dividing the time needed to consume all SCOA supplemented by the remaining time of the cycle.

It was assumed that all ammonia was consumed for growth considering that thiourea fully inhibits nitrification. Active biomass elemental composition was represented by the molecular formula $C_5H_7NO_2$ indicating that 1 mg of N is needed to produce 8 mg of active biomass.

Acetic, propionic and butyric acids volumetric consumption rates (- r_{Acet}, - r_{But}, - r_{prop}) were determined by adjusting linear functions to the experimental data for each variable concentration divided by the biomass concentration at that point over time, and calculating the first derivative at time zero. PHA production yield on substrate ($Y_{PHA/S}$) and

biomass production yield on substrate ($Y_{X/S}$) were calculated by dividing the amount of each parameter by the total amount of substrate consumed (corresponding to the sum of all SCOA).

6.3. Results and Discussion

6.3.1. SBR operation

An important aspect connected to the optimization of MMC PHA production systems, particularly from surplus-based feedstocks, relays on the ability to steer the MMC to consume the desired substrate fraction and channel it to PHA production. Using a very complex substrate, it might allow the development of an undesired side population, reducing the overall production yield (Marang et al., 2014; Queirós et al., 2016). By playing with the operational parameters, in this case, a defined SCOA profile and cycle length, an enriched PHA-accumulating culture would be expected.

To achieve a stable response to the imposed process conditions, which was verified by the reproducibility of the oxygen profiles along the cycle, the variation of feast/famine ratio was followed. A sudden increase in the O_2 concentration was observed corresponding to the depletion of most of the readily biodegradable COD substrates, in this case, the SCOA. SCOA depletion time was also considered to correspond to the length of the feast phase. However, a slower and progressive increase of O_2 took place, suggesting the consumption of other carbon sources (Valentino et al., 2015). F/F ratio is an important parameter to evaluate the culture selection process. In general, low F/F values (< 0.2) ensure physiological adaptation of the microorganisms, favoring PHA storage during the feast phase (Albuquerque et al., 2011; Reis et al., 2011). As shown in Fig. 6.1., around day 34, the F/F ratio stabilized at 0.091, which is an indication of the selection of a PHA-storing MMC. On day 44, the cycle duration was changed from 24 h to 12 h and the MMC responded with a quick stabilization despite the increase in the F/F ratio, 0.2. Notwithstanding, 0.2 value is still an indicative of the culture accumulation response (Albuquerque et al., 2010; Dionisi et al., 2007). The change of cycle duration was also followed by stabilization of SCOA consumption rates. The average acetic acid uptake rate (2.99 ± 2.02 Cmmol L^{-1} h^{-1}) was higher than the average uptake rates of propionic

acid (1.23 ± 1.13 Cmmol L^{-1} h^{-1}) and butyric acid (2.19 ± 1.65 Cmmol L^{-1} h^{-1}), which demonstrates a preference of the MMC for acetic acid. The propionic acid consumption was relatively constant throughout the SBR operational time and the butyric acid uptake rate stabilized from day 37.

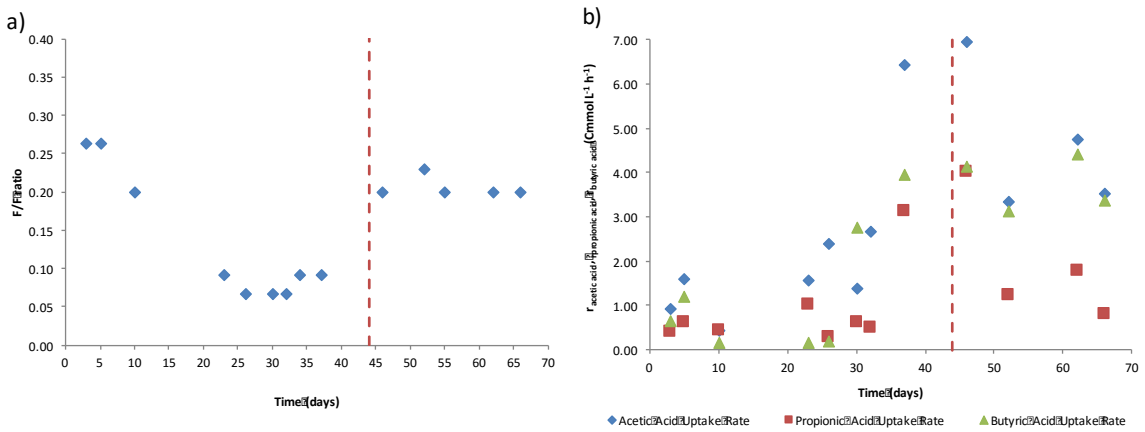

Fig. 6.1. Evolution of F/F ratios (a) and uptakes rates of acetic, propionic and butyric acids (b), along the SBR operational period. The red dashed line marked when cycle length and OLR were changed.

The increase on OLR affected directly the biomass concentration (data not shown), and indirectly the F/F ratio (an increase from around 0.1 to 0.2) keeping the SRT constant at five days. Nevertheless, if F/F value fall inside the range of PHA accumulation behavior (≤ 0.2), this could be a solid strategy to increase the overall productivity of the process as intended. In turn, lowering the SRT to even intensify the biomass production (and consequently, volumetric productivity) usually leads to a loss of storage capacity since most of the carbon is direct to energy and biomass production (Lemos et al., 2008).

There are two ways to rise OLR: by increasing either the influent substrate concentration or decreasing the HRT while keeping substrate concentration constant. The second alternative will not run the risk to inhibit the culture (HSSL is a complex and toxic feedstock) nor increase the F/F ratio drastically. In this way, since the influent substrate concentration was kept constant, the specific rate of substrate uptake would increase (Fig. 6.1), alongside with the increase of biomass concentration and, hopefully, volumetric productivities (Albuquerque et al., 2010).

Comparing the results obtained with previous works using HSSL as substrate, it is

noteworthy that the supplementation of SCOA led to a quicker stabilization of the MMC for both cycle durations: 20 days for cycles of 24 h and 5 days for 12 h. Queirós et al. (2016, 2014) using HSSL reported the inability to obtaining a stable MMC using cycles of 12 h and more than 250 days for 8 h.

Fig. 6.2. shows the evolution of Δ%PHA, active biomass and PHA concentrations along the SBR operational time. The average variation of Δ%PHA, during pseudo-stationary state from day 34, was 7.5 ± 2.4%. The values of Δ%PHA and PHA concentration were unstable throughout the first 34th days of SBR operation and several peaks of production were detected. Between the 46th and 66th day, Δ%PHA was higher than before, and PHA production became more stable and it varied in the range 9.07 ± 2.17%. This leads to believe that the culture PHA storage was not affected by change of cycle duration. Moreover, contrary to Queirós et al. (2016) that observed the production of a second storage polymer, glucose biopolymer was not detected during this work. A considerable amount of active biomass (0.4 – 1 g L^{-1}) was also observed. Such fact could probably be a sign of the existence of a side population able to consume other carbon sources without the need to accumulate any reserve substances. A similar situation was observed by Marang et al. (2014) when acclimatizing an MMC to acetic acid and methanol allowing a side population to growth without PHA accumulation due to methanol.

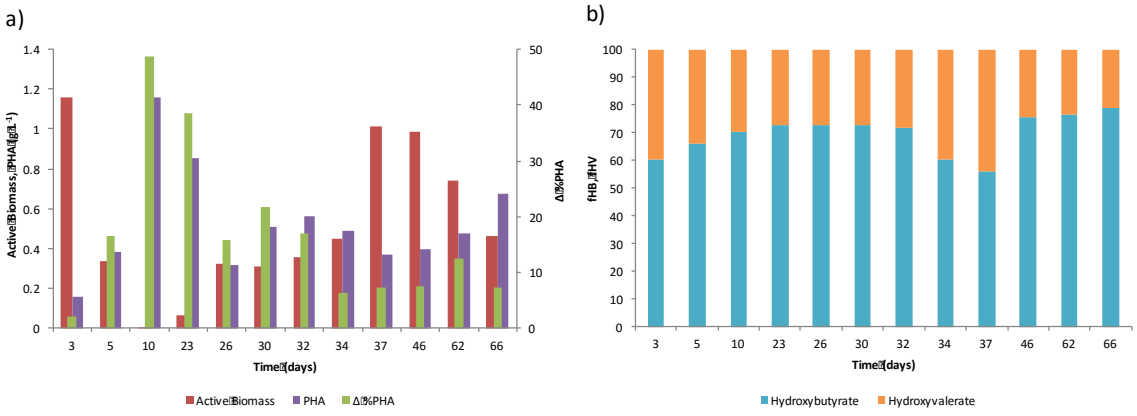

Fig. 6.2. Evolution of Δ%PHA, Active Biomass and PHA concentrations (a) and contents of 3HB and 3HV (b) throughout the SBR operational time.

Fig. 6.2 shows the evolution of the produced monomers during the SBR operation. The supplementation of HSSL with an SCOA as propionic acid in the SBR feeding resulted in the formation of P(3HB-*co*-3HV). An average monomeric composition (on a molar basis) of 69:31 ± 7:7 (3HB:3HV) was obtained throughout the SBR operational period. After the change of cycle duration on day 44, the copolymer composition became more stable, with a decrease in 3HV content, 77:23 ± 1:1 (3HB:3HV). Besides, no GB formation was observed, contrary to Queirós et al. (2016), allowing the carbon to be only used for growth and PHA accumulation. This represents an improvement relatively to the previous works with HSSL alone. Queirós et al. (2014), using the same OLR and 12-h cycle, observed the formation of P(3HB), while Queirós et al. (2016) reported the formation of P(3HB-*co*-3HV) with significant amounts of 3HV, around 20%, with high OLR (17 gCOD L^{-1} d^{-1}) and 8-h cycle and significant amounts of GB production reducing the PHA production yields. Homopolymers, such P(3HB), and copolymers, like P(3HB-*co*-3HV), can form different crystalline phases and present different physical properties. Consequently, the market application will be completely different (Laycock et al., 2013). Moreover, from the commercial point of view, P(3HB-*co*-3HV) is much more interesting than P(3HB), since it has a broader range of applications due to its improved physical and chemical properties (Philip et al., 2007). Several works already showed that the monomeric distribution of microbial PHA produced by MMC can be controlled by manipulating the SCOA composition in the feeding. A feeding with acetic acid as sole carbon source usually results in the formation of P(3HB) under ADF conditions (Lemos et al., 2006). When other SCOA, such as propionic acid, and valeric acid are added, and propionyl-CoA is formed as a precursor for PHA production, resulting in a copolymer containing 3HV monomers (Lemos et al., 2006; Tan et al., 2014).

Despite the short period of operation, the P(3HB-*co*-3HV) could be considered quite stable. This is an important aspect since the stability of the PHA composition over reactor time of operation is also highly desirable (Laycock et al., 2013). Further applications and manipulation of the polymer produced are entirely dependent on its constant composition. Moreover, a further characterization of the polymer obtained should be done to understand its physical properties.

6.3.2. SBR cycle

During the operational period, SBR was characterized through monitoring of individual cycles that allowed following several parameters, namely SCOA consumption and PHA production. Fig. 6.3 shows a representative 12-h cycle with an F/F ratio of 0.20 on day 66 of operation, the most stable period of the reactor operation.

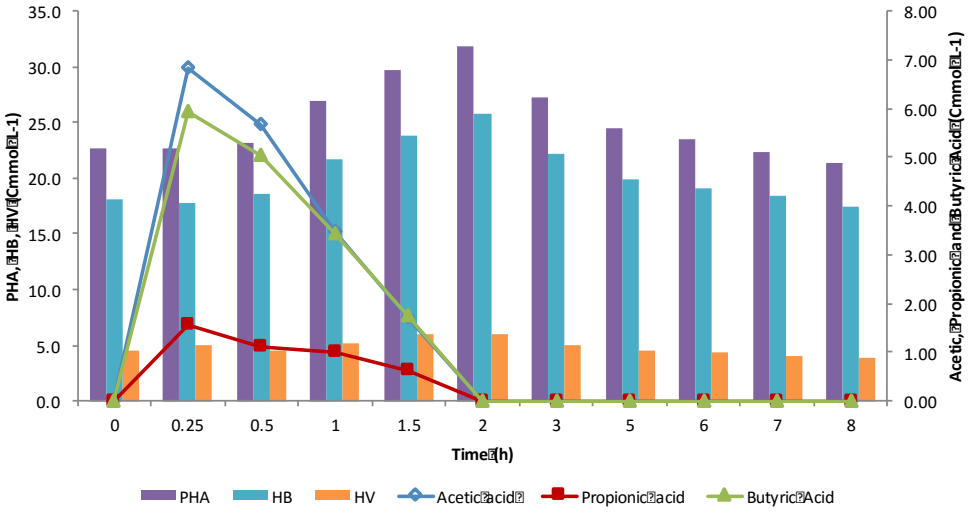

Fig. 6.3. SBR representative cycle from the 66th day of operation.

HSSL was already reported as having buffer capacities (Queirós et al., 2014) and for this reason, the SBR was operated without pH control. The pH along the cycle did not varied much, increasing from 7.8 (after feeding) to 8.8 maximum (data not shown). Chua et al. (2003) studied the effect of pH on PHA content using acetic acid as substrate. They found that, when controlling the pH at 6 or 7, the PHA content was lower than at pH 8 or 9 (Chua et al., 2003). Serafim et al. (2004) also found that the polymer yield per substrate and the intracellular P(3HB) content were higher at pH 8 than at pH 7, and increased sharply when the pH was not controlled, varying between 8.0 and 9.5. On the other hand, Oehmen et al. (2013) studied the effect of pH control on the volumetric productivity of PHA production by an MMC and verified that the operation of an SBR with pH control at 8 resulted in higher volumetric productivity of PHA than without pH control (pH 8 – 9). The culture selected at pH 8 demonstrated a higher total volumetric productivity of PHA, since it could achieve a similar specific PHA production capacity while exhibiting a faster

biomass growth rate. The buffering capacity of HSSL was an additional advantage of using it as substrate for PHA production since, apparently, no pH control would be necessary for large-scale application.

Along the cycle, acetic and butyric acids were consumed preferentially with uptake rates of 3.51 and 3.36 Cmmol L^{-1} h^{-1}, respectively. Propionic acid was consumed at 0.802 Cmmol L^{-1} h^{-1}. The three SCOA were fully exhausted during the first two hours. The same tendencies of SCOA consumption were observed in the 24 h cycle (data not shown). During the first 2 hours of the cycle, COD was consumed at 0.276 g L^{-1} h^{-1}. No tendency on LS consumption was observed remaining their concentration around 2.81 g L^{-1}. Also, there was no consumption of the two major sugar components of HSSL, xylose and glucose. This could be due to the supplementation with SCOA that allow to select a culture unable to metabolize sugar. HSSL could then be used for further bioprocesses.

The maximum PHA concentration (31.8 Cmmol L^{-1}) was registered at the 2nd hour, which corresponded to the end of the feast phase. For this cycle, Δ%PHA was 7.3% and the $Y_{PHA/S}$ was 0.64 CmmolPHA $CmmolS^{-1}$. After the 2nd hour, the concentration of PHA began to decrease since the stored PHA was consumed for growth and maintenance. The PHA concentration decreased 33% between the 2nd and 8th hour of the cycle. The composition of produced P(3HB-*co*-3HV) was uniform throughout the cycle, with a monomeric composition of 79:21 (3HB:3HV) at 2 h. The polymer composition during the cycle was more stable for 12 h cycles than for 24 h (data not shown).

This cycle started with biomass presenting a PHA content of 4.9%, meaning that a significant part of the stored PHA was never fully consumed. This was observed in most SBR cycles for both cycle durations studied. Despite, the relatively high PHA content, the MMC was able to store more PHA during the cycle.

6.3.3. Accumulation Tests

Three accumulation tests were performed in fed-batch to test the MMC storage capacity: without nutrient limitations (A), under phosphorous limitation (B), and under nitrogen limitation (C). All tests were performed using biomass collected from the 12 h pseudo-stationary phase. In tests A and C, 5 feeding pulses were given with the same

composition as the feed of the SBR, but in batch B only 3 pulses were given. Each pulse was added when a sudden increase in DO was detected, since it is usually associated with SCOA depletion.

In test A, the maximum PHA content obtained was 23.7% of cdw, after 7.75 h. The overall $Y_{PHA/S}$ got in this accumulation test was very low, 0.09 CmmolPHA $CmmolS^{-1}$. Such low value could be justified with the feeding of nitrogen and phosphorous, nutrients required for cell growth, preventing PHA accumulation. The longer the culture was exposed to a medium without limitation; more carbon deviates from PHA storage to biomass growth. Regarding the polymer stored, a slight decrease in 3HV proportion was observed, 80:20 (3HB:3HV), when compared with the selection SBR.

In test B, phosphorous limitation was imposed since it was previously reported to enforce less stress to the MMC than nitrogen. Phosphorous can be considered to be more transferable than nitrogen from one component of the cell to another, and it can decrease ATP synthase activity, resulting in a restrained Krebs cycle, promoting the conversion of excess carbon into PHA (Cavaillé et al., 2013). In test B, growth rates obtained were lower than in the previous test, achieving part of the objective to limit cell growth. The MMC obtained a maximum PHA content of 22.8% of cdw, after 4 h and produced a P(3HB-*co*-3HV) with 29% of 3HV. The overall yield $Y_{PHA/S}$ obtained in this test was 0.15 CmmolPHA $CmmolS^{-1}$. Both PHA content and production yield were relatively low when comparing with other works using real complex substrates (Albuquerque et al., 2007; Moita et al., 2014). Despite the increasing PHA content and overall $Y_{PHA/S}$, phosphorus limitation still did not reveal a robust strategy to maximize the PHA accumulation.

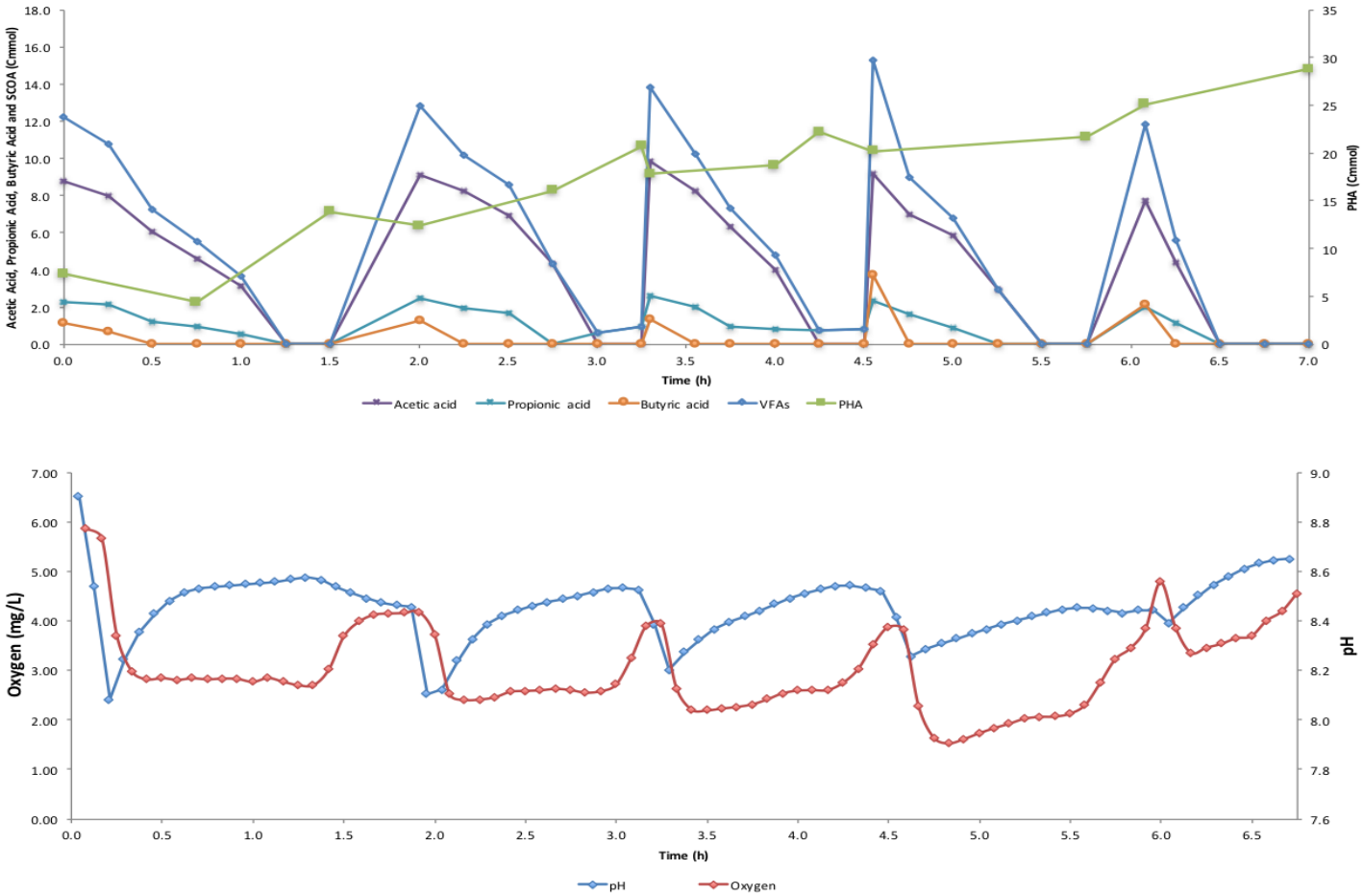

Fig. 6.4. Evolution of pH, DO, PHA, Acetic Acid, Propionic Acid, Butyric Acid and SCOA concentrations in kinetic test performed with nitrogen limitation. Test performed on the 68th day of operation.

In test C, the maximum PHA content obtained was 34.6% of cdw at the end of the test (Fig. 6.4). The overall yield $Y_{PHA/S}$ observed in this accumulation test was 0.78 CmmolPHA $CmmolS^{-1}$. The produced P(3HB-*co*-3HV) had an average monomeric composition of 76:24, near to the average composition obtained in the SBR at the same time. The imposition of nitrogen limitation led to the best PHA storage results. A considerable number of works already explored the strategy of nitrogen limitation to trigger and maximize the PHA production (Albuquerque et al., 2011; Bengtsson et al., 2008; Dionisi et al., 2005). The PHA storage capacity in accumulation test C was lower than in others described in the literature. Dionisi et al. (2005) reported a PHA storage content of 55% using olive oil mill effluents, 54% by Bengtsson et al. (2008) using paper mill effluents and a range of 56 – 77% by Albuquerque et al. (2011) from fermented molasses.

Nevertheless, in test C, a high PHA yield on substrate was obtained, 0.78 CmmolPHA $CmmolS^{-1}$, comparing to the previous works, ranging from 0.50 to 0.80 CmmolPHA $CmmolSCOA^{-1}$ (Albuquerque et al., 2007; Carvalho et al., 2014; Moita et al., 2014).

6.3.4. Microbial Community Characterization

The goal of the present study was also to establish the link between microbial population relative abundance and the performance of PHA-producing capacity of the selected MMC. The differences should be a function of the operational conditions applied and the type of substrate used. Several works that address the microbial characterization of their enrichments revealed diverse communities, due to both parameters (Queirós et al., 2015). Such characterization can contribute towards the development of process operational strategies designed to optimize the structure of the microbial community (Carvalho et al., 2014).

FISH analysis was performed to original sludge and samples collected from the SBR on days 17, 32, 45, 51, 60 and 66 of operation. Initially, specific probes for the main phyla within *Bacteria* domain were applied. The microbial community was mostly composed of

Bacteria since no *Archaea* were detected. Several FISH probes were used taking into consideration bacteria previously reported as PHA-accumulating organisms.

To identify the microbial community and to follow the MMC composition throughout the reactor operation, specific probes for the main groups within *Bacteria* domain were applied: *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Chloroflexi*, *Flavobacteria*, *Bacteroidetes*, *Sphingobacteria*, *Actinobacteria*, *Firmicutes*, *Planctomycetales*. Fig. 6.5 shows the evolution of the composition during the reactor operation. Since the beginning of the process, *Betaproteobacteria* were the dominant group and their relative abundance only suffered mild fluctuations during the whole reactor operational time. Since *Betaproteobacteria* were reported as PHA-producers, this result showed that the operational conditions applied to the SBR, favored the maintenance of this bacterial group within the MMC. *Alphaproteobacteria* were the second most dominant group in the bacterial community throughout the SBR operational time, contrary to what was described so far with HSSL (Queirós et al., 2014). This proves that a small change, the introduction of different SCOA profile, led to a different community enrichment with good PHA accumulation capacity. Using solely HSSL as substrate at high OLR and shorter cycle lengths, 17 gCOD L^{-1} d^{-1} and 8 h, respectively, a different community was selected (chapter 5). Dominated by *Alphaproteobacteria*, the PHA production was low despite the presence of a considerable number of PHA-storing microorganisms. A second preferred reserve polymer was accumulated, a glucose polymer (chapter 5).

As previously mentioned, the operational conditions were changed from 24 h to 12 h cycle on day 44. The MMC took 10 days to stabilize and reach a pseudo-stationary phase. By analyzing the bacterial composition during 12 h cycles, it is possible to verify that the bacterial community did not suffer significant changes during this period, at the class level. *Betaproteobacteria* remained the dominant group, and their relative abundance was constant during this period (42.99 ± 0.38% on day 60 and 40.76 ± 2.15% on day 66). Results obtained in this study are in line with previous studies that identified organisms belonging to *Alphaproteobacteria*, *Betaproteobacteria* or

Gammaproteobacteria classes as PHA-accumulating organisms selected under ADF (Albuquerque et al., 2013; Ferreira et al., 2015; Jiang et al., 2012; Moita et al., 2014).

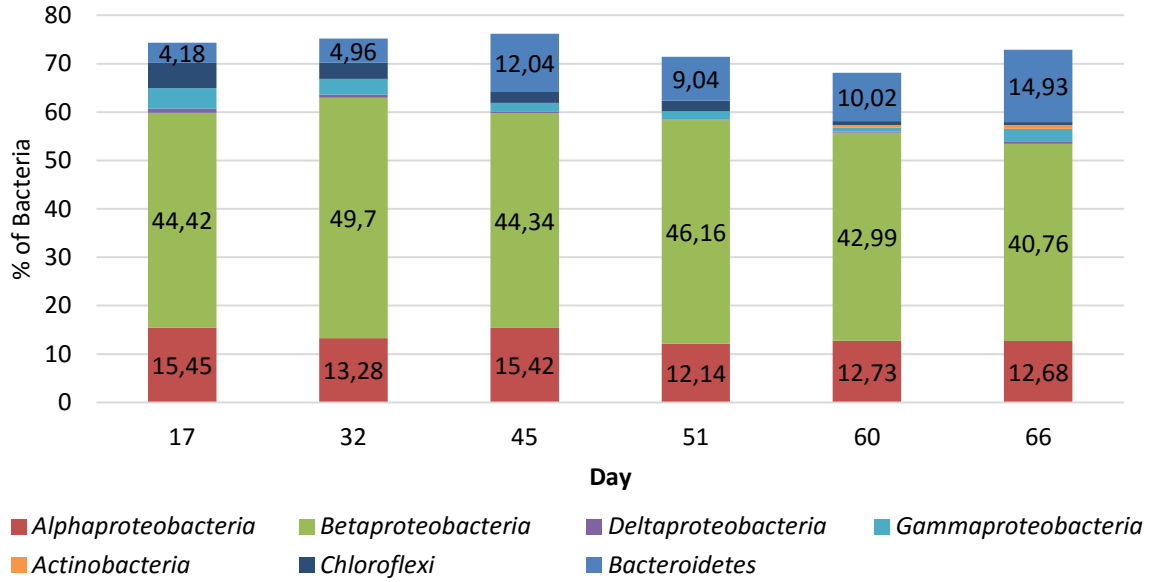

Fig. 6.5. Bacterial community evolution throughout the SBR operation.

Regarding the *Alphaproteobacteria* class, specific probes for *Amaricoccus*, *Sphingomonas*, *Defluvicoccus* and *Defluvicoccus* related Tetrad-Forming Organism (TFO-DF), all PHA-accumulating organisms, were applied. Positive results were only obtained for TFO-DF (0.78 ± 0.38%), in far less extent than in others enrichments with HSSL (Queirós et al., 2014).

For the *Betaproteobacteria* group, specific probes for *Thauera* and *Azoarcus* genera were applied due to their previous identification as PHA-accumulating organisms (Queirós et al., 2015). Positive results were obtained for both genera, but only small amounts of *Thauera* (0.72 ± 0.25%) and *Azoarcus* (0.62 ± 0.02%) were found. After applying the available specific probes for genera belonging to *Betaproteobacteria*, the main part of this population remained unidentified. Nevertheless, almost whole biomass could store PHA as shown by Nile Blue staining (data not shown).

To identify the main bacteria responsible for PHA accumulation in the selected MMC, a 16S rRNA gene clone library was constructed. The sample used to extract the DNA was collected from the SBR on the 66th day of operation. 60 clones were chosen for

partial sequencing. The obtained 16S rDNA partial sequences were then analyzed by BLAST and grouped by genera. After analyzing the results of the partial sequencing, 15 clones were chosen for complete sequencing and refinement of their taxonomic affiliation. The major part of the clones presents in the clone library belonged to *Betaproteobacteria* class. These results were expected since, during the pseudo-stationary phase, *Betaproteobacteria* was the dominant group of the bacterial community.

The representatives of the fifteen most abundant clones are shown in Table 6.2 and their phylogenetic relations are presented in Fig. 6.7. Almost all of them are known PHA producers previously found in MMC selection processes for PHA production (Queirós et al., 2015).

During the analysis of 16S rRNA gene sequences, six of the 15 sequences were related to *Acidovorax*. Moreover, *Acidovorax* was previously shown to be capable of PHA storage (Schulze et al., 1999). Positive results for *Acidovorax* were obtained for all the screened samples and its content in the microbial community increased from 2.62 ± 0.93% (in day 17) to 28.98 ± 2.51% (in day 66). During the pseudo-stationary phase, the *Acidovorax* genus became the major constituent of *Betaproteobacteria,* composing 71% of this class population on day 66 (Fig. 6.7).

Fig. 6.6. Phylogenetic tree based on full-length nucleotide sequences of 16S rRNA gene of clones CR1-15 (in the tree preceded by a filled red dot). Molecular Phylogenetic analysis by Maximum Likelihood method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 42 nucleotide sequences. Evolutionary analyses were conducted in MEGA6.

Table 6.2. Results of the complete sequencing.

Sequence name	Highest Similarity	% Identity	Accession number
CR1	*Acidovorax caeni*	94%	KT262954
CR2	*Acidovorax wautersii*	98%	KT262955
CR3	*Fluviicola taffensis*	92%	KT262951
CR4	*Leadbetterella byssophila*	95%	KT262956
CR5	*Comamonas testosteroni*	90%	KT262952
CR6	*Paracoccus siganidrum*	98%	KT262957
CR7	*Agrobacterium tumefaciens*	99%	KT262958
CR8	*Acidovorax radicis*	98%	KT262959
CR9	*Simplicispira metamorpha*	98%	KT262960
CR10	*Comamonas testosteroni*	99%	KT262961
CR11	*Alcaligenes aquatilis*	94%	KT262962
CR12	*Shinella zoogloeoides*	99%	KT262953
CR13	*Pseudoxanthomonas kaohsiungensis*	99%	KT262963
CR14	*Acidovorax delafieldii*	98%	KT262964
CR15	*Simplicispira metamorpha*	97%	KT262965

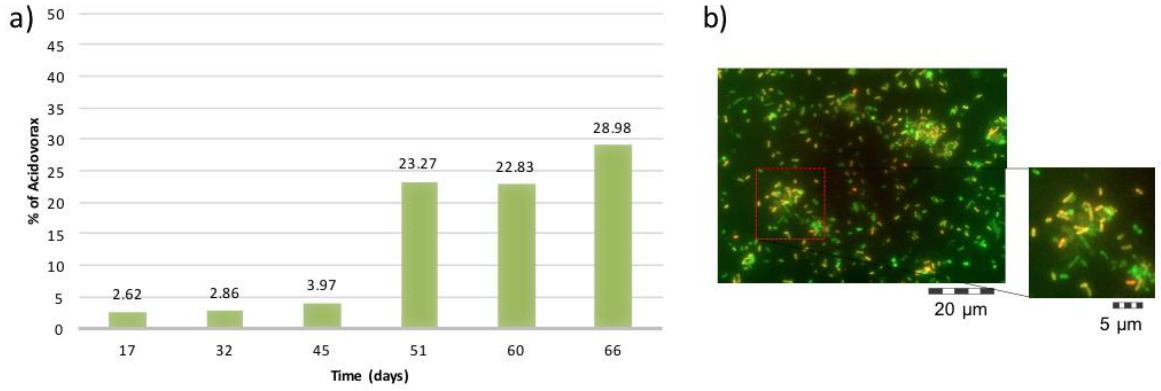

Fig. 6.7. a) *Acidovorax* content evolution throughout the reactor operation. Percentages are about the relative abundance of *Betaproteobacteria.* b) FISH picture of *Acidovorax* on day 66. Green cells are hybridized with EUBmix and the yellow cells also hybridized with ACI145 probe.

Clones related to *Comamonas* spp. and *Novosphingobium* spp. were also detected. Ferreira et al. (2016) isolated and characterized organisms able to store PHA, from an MMC selected under the same conditions as the ones of this study, same OLR and 12 h cycle length, but using non SCOA-supplemented HSSL. Without this supplementation, the culture was dominated by microorganisms belonging to

Alphaproteobacteria. This observation suggested that the simple addition of known SCOA precursors of PHA lead to marked differences in the microbial community composition.

Several microorganisms present in the clone library were already described as PHA-accumulating organisms in pure culture: *Comamonas acidovorans* using 1,4-butanediol and glucose (Lee et al., 2004), *Paracoccus denitrificans* consuming ethanol and *n*-pentanol (Chanprateep et al., 2001), *Cupriavidus necator* using soya wastes from a soya milk dairy (Doi et al., 1990) and *Alcaligenes latus* using malt wastes from a beer brewery plant as substrate (Yu et al., 1999).

There were also sequences related to *Fluviicola taffensis*, *Leadbetterella byssophila*, *Paracoccus siganidrum*, *Agrobacterium tumefaciens*, *Alcaligenes aquatilis*, *Shinella zoogloeoides* and *Pseudoxanthomonas kaohsiungensis*. Other clones had phylogenetic relationships with *Lampropedia hyalina*, *Azoarcus* sp., *Thauera* sp., *Bacillus megaterium*, *Plasticicumulans acidivoran*s, *Thiocystis violacea* and *Zoogloea* sp..

6.4. Conclusion

The supplementation of HSSL with different SCOA reduced greatly the adaptation period of the culture and allowed to produce a copolymer of P(3HB-*co*-3HV) without GB accumulation.

A pseudo-stationary state was reached after 34 days of SBR operation, which indicated that the MMC could successfully adapt to the different conditions imposed. A stable PHA accumulating MMC was obtained with a low organic loading rate and with no ammonia limiting conditions, thus providing the culture with the selective pressure for PHA accumulation. OLR and cycle length were also shown to have affected the kinetics of substrate consumption and the type of polymer stored. Substrate uptake rates and polymer production rates were found to increase with increasing substrate concentrations in the selection reactor, without the consumption of any HSSL's sugars. For the accumulation step, the presence of nitrogen showed to negatively affect the PHA production in the accumulation step. Under ammonia limitation the culture reached 34.6% P(3HB-*co*-3HV) cdw.

FISH analysis revealed a dominant *Betaproteobacteria* class, around 41% since the beginning of the reactor operation. *Acidovorax*, a PHA producer, was the dominant genus, overcoming the remaining population when the OLR and cycle length were changed. Furthermore, clone library results presented several bacteria that were already described as PHA-storing microorganisms.

Introducing a pre-fermentation step of HSSL would be the next step to be tested in the selection of PHA-producing MMC. By testing different operational conditions during the acidogenic fermentation, different mixtures of SCOA would be obtained and the impact of feeding these mixtures on the selection step should be evaluated.

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Chapter 7

Enhancement of bioprocessing of HSSL: adsorption of microbial inhibitors

Agroforestry wastes and by-products are now being tested as substrates to produce value-added products. This approach is meant to be included in a lignocellulosic biorefinery concept contributing to increase the sustainability and profit of agriculture, wood and pulp and paper industries. However, such wastes and by-products usually possess, a high content of toxic and recalcitrant components that can act as inhibitors of microbial processes. Consequently, a pre-treatment step is usually required prior to fermentation. Three adsorbents, activated carbon, chitosan and bentonite, were tested, for the first time, to treat HSSL. This work intended to remove the major fraction of LS from HSSL.

The study focused on optimization of the amount of adsorbent and the pH value that led to the best removal of LS. In this way, the best LS removal yield (gLS removed $gAdsorbent^{-1}$) during preliminary assays were 2.91, 2.73 and 3.12 for 25 g L^{-1} of bentonite at pH 4.60, 10 g L^{-1} activated carbon at pH 2.00 and 20 g L^{-1} chitosan at pH 2.00, respectively. In terms of percentage, the LS removed was 43.8% for bentonite, 41.1% for activated carbon and 37.6% for chitosan.

The removal of two components of interest was also monitored, xylose and acetic acid. Activated carbon revealed to be the less selective, removing 17.4% and 22.3%, respectively, while with bentonite the lowest removals of these compounds were observed, removing 4.7% and 12%.

7.1. Introduction

European biobased economy already approves a large part of materials to be generated from several by-products and wastes (Fava et al., 2015). Ultimately, the objective is to create a biorefinary able to house several technological platforms to valorize as much wastes and by-products as possible.

Agroforestry wastes and by-products are now the core of new business models and strategies. This approach aimed making agriculture, wood and pulp and paper industries sustainable holdings (Devappa et al., 2015). Their residues can be a cheap and abundant source, onsite, of innovative chemical, biomaterials and substrates for biotechnological production, allowing a revenue generation (Rafione et al., 2014). Research is focused on developing new technologies able to use low-value substrates and thus reducing the overall operating costs. Important building blocks as succinic acid are being produced from corncob hydrolysates (Yu et al., 2010); SCOA from olive oil mill effluents (Bertin et al., 2010); several biofuels such biohydrogen from sorghum (Nagaiah et al., 2015) and bioethanol from HSSL (Pereira et al., 2012); PHA from olive mill effluents (Valentino et al., 2014), from paper mill wastewaters (Jiang et al., 2012) or from HSSL (Queirós et al., 2014). These are some examples of a whole range of biobased products obtained from wastes and by-products (Koutinas et al., 2014).

Nonetheless, a considerable amount of processes that uses these substrates struggles with low yields and productivities of the desire products. Compounds like acetic, formic and levulinic acids, furfural, aromatic compounds (phenols), soluble lignin and extractives components act as inhibitors on microbial growth and therefore a pre-treatment step is usually necessary prior to fermentation (Koutinas et al., 2014; Pereira et al., 2012). Such inhibitors are often present in spent sulfite liquors leading to difficulties in establishing bioprocesses like bioethanol (Pereira et al., 2013; Pereira et al., 2012) and PHA productions (Queirós et al., 2015, 2014). Applying ion exchange resins, it was possible to fraction components from HSSL, separating lignosulphonates and phenolic (LS) components (originated from lignin) from the sugar fraction. However, such process is expensive and could hinder further bioprocesses (Fernandes et al., 2012).

Other materials like clays, such bentonite, are naturally abundant and are receiving a lot of attention as potential adsorbents due to their cost effectiveness, environmentally stability, high adsorption and ion exchange properties (Szabó et al., 2014). Activated carbon is another material that presents large surface areas, porous micro nature, large adsorption capacity, high purity and good accessibility. However, activated carbon is not very selective and it is expensive when compared to other adsorbents (Nam et al., 2014; Tran et al., 2015). Another promising material is chitosan. Derived from chitin, it is easily found or extracted from the shells of some sea foods. Its sorption capacity is determined by contents of amino and hydroxyl functional groups in chitosan. Nitrogen chitosan is in the form of the primary amine that could suffer typical reactions of amines such as N-acetylation. This compound is very reactive, enabling the achievement of many derivatives (Saitoh et al., 2011; Tran et al., 2015).

All the materials above described have their sorption capacity tightly related to pH, temperature and contact with the compounds to be removed (Banat et al., 2000; Giraldo and Moreno-Piraján, 2014; Hameed and Rahman, 2008; Roostaei and Tezel, 2004; Saitoh et al., 2011, 2009). So, the influence of these parameters on the sorption process should be studied on the removal of phenolic compounds and their derivatives.

This work intended to study the use of bentonite, activated carbon and chitosan on the removal of phenolic compounds and their derivatives from HSSL and the effect of adsorbent concentration and pH on the adsorption process. As an attempt to infer which phenolic components were preferentially extracted by the best adsorbent, GC-MS analyses were performed.

7.2. Materials and Methods

7.2.1. HSSL

HSSL from magnesium based acidic sulfite pulping of *Eucaliptus globulus* was supplied by Caima – Indústria de Celulose S.A. (Constância, Portugal). Pre-evaporated HSSL was collected from an inlet evaporator of a set of multiple-effect evaporators to avoid the presence of free SO_2 in liquor. A first pre-treatment was applied to HSSL to remove most of the recalcitrant compounds. The pre-treatment started by a pH

adjustment to 7.0 with 6M KOH, followed by aeration with compressed air (6 hours per liter of HSSL – 6 h L^{-1}) (Pereira et al., 2013). Then, the liquor was centrifuged for 1 h at 5000 rpm. The precipitated colloids were filtered off using a 1 µm glass microfiber filter (Xavier et al., 2010).

7.2.2. Adsorption assays – Preliminary tests

Three different adsorbents were tested for the removal of LS: bentonite (VWR: CAS 1318-93-0), activated carbon (VWR: CAS 7440-44-0) and chitosan (VWR: CAS 9012-76-4). For each adsorbent three concentrations and three different pH were tested (Table 7.1). The pH was adjusted using concentrated solutions of HCl (10% w/w).

The adsorbent concentrations were chosen accordingly with literature (Banat et al., 2000; Gupta et al., 2014; Hameed and Rahman, 2008; Saitoh et al., 2011, 2009) and considering that HSSL lignosulphonates content was ≈ 166 g L^{-1}. The concentrations chosen for bentonite were 25.0, 50.0 and 75.0 g L^{-1} (Banat et al., 2000), for the activated carbon were 10, 25 and 37.5 g L^{-1} (Giraldo and Moreno-Piraján, 2014; Roostaei and Tezel, 2004) and for chitosan (85% deacetylated) were 20.0, 50.0 and 70.0 g L^{-1} (Saitoh et al., 2011). A control assay was also made with only HSSL and pH of 7.0. Additionally, a blank assay with water was made instead of HSSL to test the adsorbent influence in the spectrophotometric measurement.

Table 7.1. Conditions used for the treatment of HSSL, preliminary tests.

Conditions	Bentonite	Activated Carbon	Chitosan (85%)
Temperature (°C)	Room Temperature	Room Temperature	Room Temperature
Type of agitation	Orbital	Magnetic Stirring	Magnetic Stirring
Concentration (g L^{-1})	25.0	10.0	20.0
	50.0	25.0	50.0
	75.0	37.5	70.0
pH	2.00	2.00	2.00
	4.60	4.60	4.60
	6.80	6.80	6.80

The assays were conducted in Erlenmeyers of 250 mL under magnetic stirring and at room temperature. Bentonite assays, for logistics reasons, under orbital agitation. 2 mL samples were taken in defined intervals (1 h, 2 h, 4 h, 5 h, 6 h, 24 h, 26 h, 28 h and 48 h). Then samples were centrifuged at 13000 rpm for 10 minutes and the solid discarded. For each sample, the content of LS, xylose, and acetic acid was measured. For the assays with higher LS removal the total COD was also measured. After analyzing the preliminary data obtained, all assays were adjusted to 24 h, since the removal did not vary after this period.

7.2.3. Adsorption assays – Optimization of conditions

To optimize the adsorption process, concentrations of 50.0 g L^{-1}, 25.0 g L^{-1} and 70.0 g L^{-1} were chosen for bentonite, activated carbon and chitosan suspensions, respectively. A control assay was also made using only HSSL with pH of 2.00 (Table 7.2).

Table 7.2. Optimal conditions for LS removal in HSSL used for optimized tests.

Adsorbents	Bentonite	Activated Carbon	Chitosan
Temperature (°C)	Room Temperature	Room Temperature	Room Temperature
Type of Agitation	Paddles	Paddles	Paddles
pH	2.00	2.00	2.00
Concentration (g L^{-1})	50.0	25.0	70.0

The adsorption process was performed in 1 L flasks with mechanical agitation of 300 rpm using *Yellowline OST 20 digital* rotor. Samples of 2 mL were taken at specific times:1 h, 2 h, 4 h, 5 h, 6 h and 24 h after the beginning of the treatment. Then samples were centrifuged and the pellet discarded. The supernatant was used for the analysis of LS, COD, xylose and acetic acid content.

7.2.4. Analytical Methods

LS content was quantified by spectrophotometry at 273 nm (Restolho et al., 2009). The absorbance was chosen according with the absorption specter of lignin. Samples

were diluted 1:1990. The LS concentration was calculated accordingly with the Beer-Lambert law, with a molar absorptivity of 7.41 g^{-1} cm^{-1} (Xavier et al., 2010).

Soluble COD (COD_{sol}) was measured with a Merck's spectrophotometer kit, at 605 nm, using solutions prepared accordingly with the *Standart Methods* (Clesceri et al., 1998). Samples were diluted 1:247.5. The absorbance values obtained were converted to COD concentration through a calibration curve with glucose and potassium hidronoftalate standards in the range of 0 – 1 g L^{-1}.

Concentrations of xylose and acetic acid were determined by HPLC. Samples were diluted 1:10 and 600 µL were filtered using a membrane of 0.2 µm at 10000 rpm for 20 min. They were injected (auto sampler – HITACHI L-2200) in an ion exchange column Aminex HPX-87H, connected to a pump (HITACHI L-2130) and refractive index detector HITACHI L-2490. The column temperature was 40 °C (Oven Gecko 2000) and the eluent was H_2SO_4 0.01 N at a flow rate of 0.6 mL min^{-1} at room temperature. A calibration curve was obtained with standards of xylose (0 – 3 g L^{-1}) and acetic acid (0 – 1.5 g L^{-1}) (Xavier et al., 2010).

For the phenolic compounds analysis, 20 mL of samples of assays with bentonite (25.0, 50.0 and 75.0 g L^{-1} at pH 2.00 and 4.60) were acidified until pH 2 with concentrated HCl. Three liquid–liquid extractions with diethyl ether (1:1, v/v) were applied to each sample. The organic phase was collected and passed through anhydrous sodium sulphate to remove traces of aqueous phase. Then, the collected organic phase was concentrated on a vacuum rotary evaporator at 40 °C until complete dryness. The remaining solids were dissolved in 0.5 mL solution of pyridine with 2 mg mL^{-1} of tetracosane, acting as internal standard (IS), 200 µL of BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) and 50 µL of TMSC (trimethylchlorosilane) were added and the mixture was left to react at room temperature during 24 h (Marques et al., 2009). Then, samples were analyzed as trimethylsilane (TMS) derivatives in a gas chromatograph (Trace GC 2000 series) coupled to a mass spectrometer, GC–MS (Finnigan Trace MS mass spectrometer). The chromatographic conditions were as follows: initial temperature 80 °C for 5 min; temperature rate 4 °C min^{-1}; final temperature 260 °C for 10 min; injector temperature 290 °C; detector temperature 290 °C. Identification of chemical compounds was made

using MS spectral library or based on the comparison of their retention time and the results obtained were related with the amount of internal standard added to samples.

7.2.5. Calculations

The following equations were used to determine the percentage of LS removed (% removal) and the yield on LS removed by amount of adsorbent ($Y_{removal}$):

$$\%\text{LSremoval} = \frac{(\text{LSincontrol} - \text{LSinthesample}) \times 100}{\text{LSinthecontrol}} \quad (7.1)$$

$$LS\ removal\ yield = \frac{LS\ removed}{Adsorbent\ concentration} \times 100 \quad (7.2)$$

For GC-MS compound identification and calculation of weight:

$$\text{Weight(mg)} = \frac{(\text{peakarea(mm)} \times (1.98(\frac{\text{mg}}{\text{mL}}) \times 0.5\text{mL})}{\text{Tetracosane(peakarea)}} \quad (7.3)$$

$$\%(w/w) = \frac{weight\ (mg)}{total\ compound\ ext.\ weight\ (mg)} \times 100 \quad (7.4)$$

7.3. Results and Discussion

7.3.1. Preliminary assays

7.3.1.1. Bentonite

Bentonite was tested as adsorbent in the adsorption process of HSSL components in three concentrations, 25.0, 50.0 and 75.0 g L^{-1}, at three different pH values, 2.00; 4.60 and 6.80 with a blank assay with water instead of HSSL. The blank assay allowed to inferred that for all adsorbents there was no effect on the spectrophotometric method used to measure LS content.

The adsorption assays had the duration of 48 h. However, after analyzing the first results obtained the duration was adjusted to 24 h. The results suggested that the equilibrium was established in the solution before 24 h. This was also observed by Banat et al. (2000) in an assay of phenol adsorption by bentonite when the equilibrium was

reached at 6 h of contact (Banat et al., 2000). Table 7.3 and Fig. 7.1 show that a higher LS adsorption was obtained, in the 24 h assay at pH 4.6 and with a concentration of 25.0 g L^{-1} (43.8% removal), corresponding to a removal yield ($Y_{removal}$) of 2.91 gLS $gAdsorbent^{-1}$ (Table 7.3). With 50 g L^{-1} of bentonite, the highest removal was 22.8% and with 75.0 g L^{-1} was 32.5%, both at pH 2.00.

Bentonite possesses, mostly, a negative charge, due to anions in its surface, most of them with oxidative capacity. At the extremities of the layers, bentonite possesses some cations like iron, magnesium and aluminum, resulting in localized positive charges (Stupp et al., 1999). Therefore, LS and phenol adsorption by this adsorbent should be better at lower pH, which was confirmed by the better removal yields obtained at acidic pH. This effect could be attributed to the ionization of phenolic compounds at higher pH. Phenolic compounds are weak acids (pKa = 9.89) that ionize at higher pH, resulting repulsive forces between the phenols and adsorbent (Banat et al., 2000). Nonetheless, Beccari et al. (2001) observed the inverse tendency, obtaining a higher phenol removal at pH 8.0 not providing, however, any explanation for the observation (Beccari et al., 2001).

The ionic fraction of phenolate ions, caused by the phenolic compound ionization can be calculated according with equation 5, which shows that the concentration of these ions increases with the pH:

$$\varphi_{ions} = \frac{1}{\left[1 + 10^{(pKa - pH)}\right]} \qquad (7.5)$$

So, by keeping the pH values low the repulsion forces between the negatively charged bentonite and phenolate ions are minimize, promoting the adsorption of the compounds.

Regarding the adsorbent concentration, Al-Asheh et al. (2003) observed a decrease in phenol concentration when a higher adsorbent concentration was used. The increase of the contact area between adsorbent and phenolic compounds could explain this behavior. The results in Fig. 7.1 do not show this tendency, since higher removals were observed at lower adsorbent concentrations. During the assay, the formation of

adsorbent granules derived from insufficient agitation was observed. These granules could be responsible for diminishing the contact area between the bentonite and components of HSSL resulting in a lower adsorption yield even with higher concentrations of adsorbent (Table 7.3).

7.3.1.2. Activated carbon

Activated carbon is an adsorbent used in diverse applications because of its high superficial area and microporous structure. However, it is not very selective so it can adsorb not only phenolic compounds but also other compounds present in HSSL (Navarro et al., 2006). Using activated carbon, LS removal was highest at pH 2.00, for all concentrations studied (Table 7.3, Fig. 7.1). At pH 2.00 and 10.0 g L^{-1}, a LS removal of 22.4% was obtained, while at 25.0 and 37.5 g L^{-1} a removal of 41.1 and 38.6% was achieved. These results were confirmed by other researchers that also tested the adsorption of phenolic compounds by activated carbon, observing higher adsorption at lower pHs and an increase of phenol adsorption with the increase concentration of activated carbon (Gupta et al., 2014; Hameed and Rahman, 2008). Activated carbon is composed by several layers of graphite, having in its surface functional groups including carbonil, carboxyl, quinone and lactone containing oxygen at the surface, which gives it the character of Bronsted base (Stavropoulos et al., 2008). Similar to bentonite, the higher phenol adsorption by activated carbon at lower pH can be explained by the phenol ionization at pH values under the pK_a and by the electrostatic repulsions between adsorbent surface and the anions formed at higher pH values (Hameed and Rahman, 2008).

From Fig. 7.1, it is also possible to verify that LS adsorption was favored with higher adsorbent concentrations (37.5 g L^{-1}) for all tested pH. However, using 25 g L^{-1} of activated carbon at pH 2.00, 62.2 g of COD were removed, corresponding to a decrease of 32.7%, like the removal observed using 37.5 g L^{-1} of activated carbon. Regarding acetic acid and xylose contents, a significant fraction was also removed, 22.5% and 31.5% respectively.

Table 7.3. LS removal yield for the pre-treatment of HSSL.

Conditions	$Y_{Removal}$ (gLS removed gAdsorbent^{-1})	LS removal (%)
Bentonite 25.0 g L^{-1}; pH 2.00	0.09	1.4
Bentonite 25.0 g L^{-1}; pH 4.60	2.91	43.8
Bentonite 25.0 g L^{-1}; pH 6.80	0.57	8.6
Bentonite 50.0 g L^{-1}; pH 2.00	0.76	22.8
Bentonite 50.0 g L^{-1}; pH 4.60	0.00	0.0
Bentonite 50.0 g L^{-1}; pH 6.80	0.42	12.7
Bentonite 75.0 g L^{-1}; pH 2.00	0.72	32.5
Bentonite 75.0 g L^{-1}; pH 4.60	0.23	10.3
Bentonite 75.0 g L^{-1}; pH 6.80	0.00	0.0
Activated Carbon 10.0 g L^{-1}; pH 2.00	3.72	22.4
Activated Carbon 10.0 g L^{-1}; pH 4.60	0.35	2.1
Activated Carbon 10.0 g L^{-1}; pH 6.80	0.24	1.4
Activated Carbon 25.0 g L^{-1}; pH 2.00	2.73	41.1
Activated Carbon 25.0 g L^{-1}; pH 4.60	0.00	0.0
Activated Carbon 25.0 g L^{-1}; pH 6.80	0.93	14.0
Activated Carbon 37.5 g L^{-1}; pH 2.00	1.71	38.6
Activated Carbon 37.5 g L^{-1}; pH 4.60	0.52	11.7
Activated Carbon 37.5 g L^{-1}; pH 6.80	1.06	23.9
Chitosan 20.0 g L^{-1}; pH 2.00	3.12	37.6
Chitosan 20.0 g L^{-1}; pH 4.60	1.36	16.3
Chitosan 20.0 g L^{-1}; pH 6.80	2.77	33.4
Chitosan 50.0 g L^{-1}; pH 2.00	1.78	53.7
Chitosan 50.0 g L^{-1}; pH 4.60	1.73	52.2
Chitosan 50.0 g L^{-1}; pH 6.80	1.51	45.7
Chitosan 70.0 g L^{-1}; pH 2.00	1.47	62.1
Chitosan 70.0 g L^{-1}; pH 4.60	1.16	48.8
Chitosan 70.0 g L^{-1}; pH 6.80	0.96	40.6

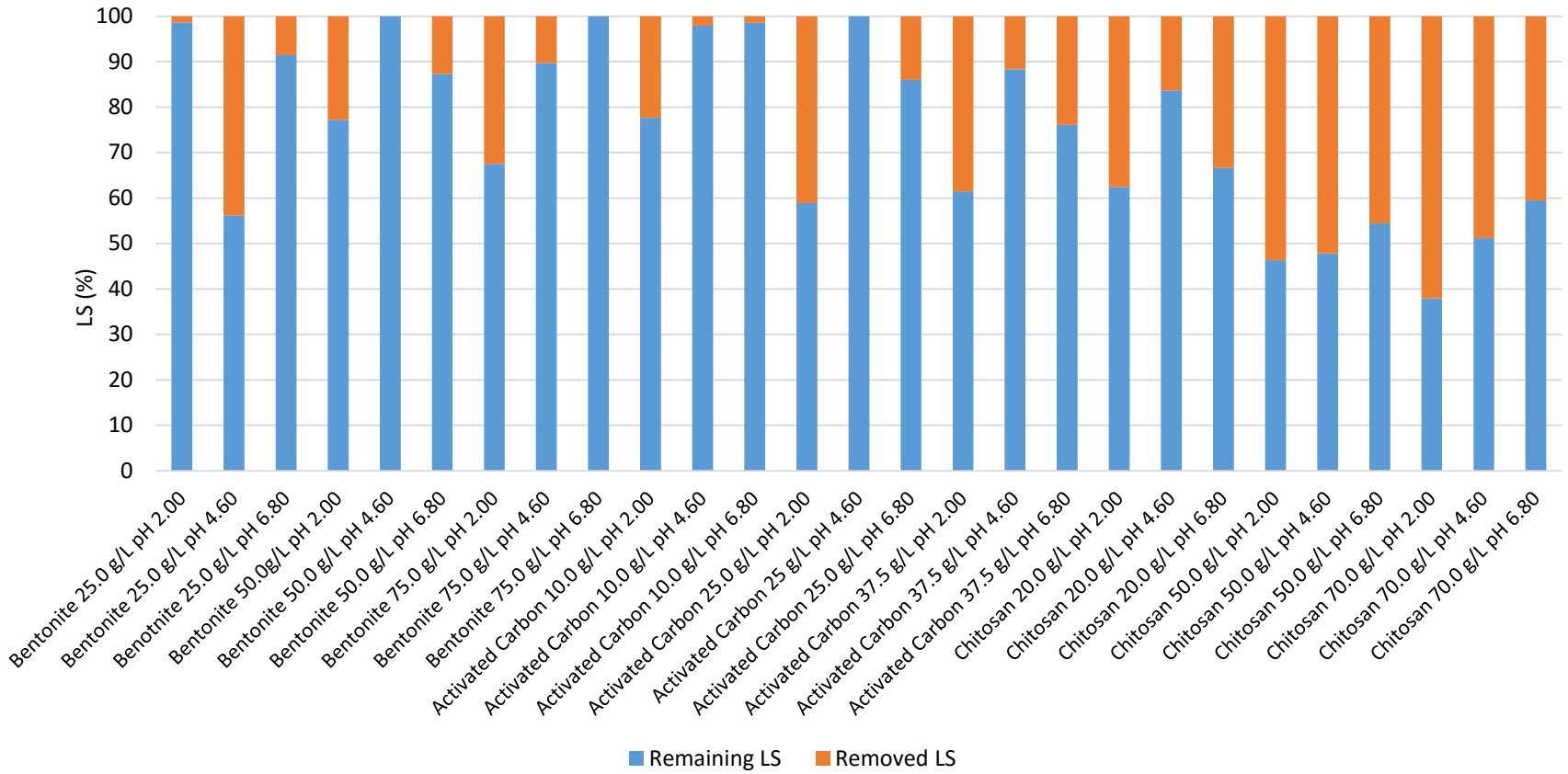

Fig. 7.1. LS removed with the three different adsorbents used in the pre-treatment. In the figure, the parameters are presented as concentration (g L^{-1})/pH.

The more accentuated decrease in xylose and acetic acid concentrations using activated carbon as adsorbent can be explained by its lack of selectivity, and the lower size of these molecules, facilitating their mass transfer to the adsorbent surface. There are, actually, several works that explore this characteristic, the capacity of activated carbon to remove acetic acid and xylose (Carvalho and Carvalho, 2004; Cruz et al., 2008; Dina et al., 2012). The removal of xylose and acetic was not even higher than the observed, probably due to the accentuated concentration of LS that quickly saturated the adsorbent.

7.3.1.3. Chitosan

Chitosan is a heteropolymer that results from the n-deacetylation of chitin (poli[β-(1-4)-2-acetamide-2-deoxi-D-glucopiranose]). Chitin is obtained from the shell of crustacean and exoskeleton of bugs and fungus mycelia.

At pH 2.00 the highest chitosan concentrations, 50.0 and 70.0 g L^{-1}, resulted in the highest LS removals, 53.7% and 62.1%, corresponding to $Y_{removal}$ of 1.78 and 1.47 gLS removed $gAdsorbent^{-1}$, respectively (Fig. 7.1, Table 7.3). The increase in LS removal with the increase on adsorbent concentration was also observed by Saitoh et al. (2009) when testing chitosan conjugated with polymers for phenolic removal (Saitoh et al., 2011, 2009). At lower pH values, chitosan has its amino free groups in a protonated state, so the anionic compounds, like phenols, are attracted. This explains the higher LS removal with higher adsorbent concentration.

As observed for bentonite and activated carbon the COD content decreased. In the assay with higher LS removal, the COD valued decreased from 217.0 g L^{-1} to 172.0 g L^{-1}, corresponding to a reduction of 20.4% in the COD content. Regarding xylose and acetic acid concentrations, chitosan was also able to adsorb those compounds resulting in a decrease of 20.9% and 25.3%, respectively.

7.3.1.4. Comparing the performance of each adsorbent

The results obtained in preliminary assays, summarized in Table 7.3, showed that the best $Y_{removal}$ were obtained for 25.0 g L^{-1} of bentonite at pH 4.60, 10 g L^{-1} of activated

carbon at pH 2.00 and 20 g L^{-1} of chitosan at pH 6.80, being 2.91, 3.72, 3.12 gLS removed $gAdsorbent^{-1}$, respectively. These results showed that although the total amount of LS removal (% LS removal) was maximum for the highest adsorbent concentrations, the $Y_{removal}$ were better when lower concentrations of adsorbent were used. This suggests that higher quantities of adsorbent probably diminish the contact between the adsorbent and the components of HSSL resulting in less adsorption. Such fact could be associated to an apparent inefficient agitation when higher adsorbents concentrations were used. Next, the assays with best total LS removals (%) results of each adsorbent were reproduced with higher volume of HSSL (1 L) and resorting to paddles agitation to overcome the previous problem reported.

For future applications, such as the use of mixed microbial cultures for PHAs production (Queirós et al., 2016), bioethanol production by yeasts (Pereira et al., 2013) or even the production of volatile fatty acids, acetic acid and xylose are some of the preferred carbon sources for such bioprocesses. The observed decreases, 22.5% for acetic acid and 32.7% for xylose, with activated carbon and 20.9% and 25.3%, respectively, with chitosan represent significant losses. Depending on the organic load used for this processes, such degree of removal of these components could hamper further bioprocesses.

7.3.2. Adsorption assays – Optimization of conditions

The assays were performed using the three adsorbents tested in the preliminary assays: bentonite, activated carbon, and chitosan. For each adsorbent were chosen the conditions that led to the best LS removal (%). Each test was performed using 1 L of HSSL at 220 rpm imposed by mechanical paddle stirring at room temperature (Table 7.2). By changing the agitation strategy, it was intended to improve the contact between adsorbents and HSSL's components overcoming the problems described in the previous section. For bentonite, three additional assays were made at pH 2.00 for the three concentrations tested in the preliminary assays to confirm the results obtained since granules were observed in the suspension. The results obtained are shown in Fig. 7.2.

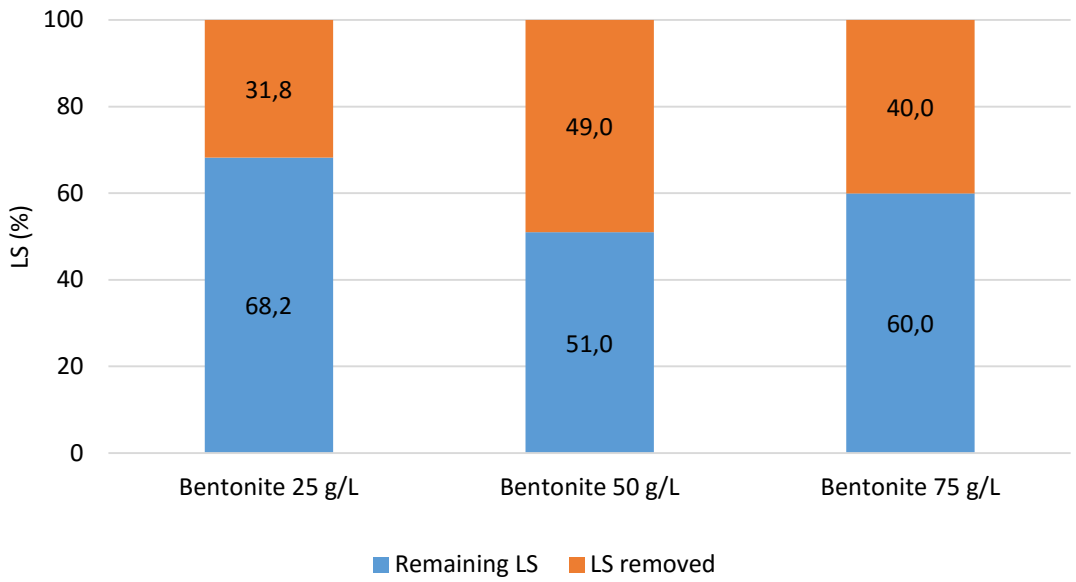

Fig. 7.2. Comparison between the LS removal yields for Bentonite for each tested concentration at pH 2.00.

It is possible to observe that the best assay was the one with 50.0 g L^{-1} of bentonite, and no longer the assay with 25 g L^{-1} at pH 4.6. Such results confirm that the type of agitation has a strong influence on the LS adsorption to bentonite.

Fig. 7.3 shows the results for the last HSSL batches treatment, for each adsorbent.

The best LS removal was obtained for chitosan with 2.0 pH and 70.0 g L^{-1} of adsorbent, 52.1% of LS removed. bentonite (50 g L^{-1}) and activated carbon (25 g L^{-1}) led to removals in line with previous tests, 38.3 and 47.8, respectively.

Regarding, xylose and acetic acid, activated carbon was the adsorbent that led to higher decrease in these compounds, 17.4% and 22.3%, respectively. These results are in line with previous observations that activated carbon is not a selective adsorbent (Abdelkreem, 2013; Kulkarni et al., 2013).

On the other hand, chitosan removed 4.7% and 10.2% of acetic acid and xylose, respectively, while bentonite removed 4.7% and 12%, respectively. These are desirable results once it showed that the used bentonite as an adsorbent treat HSSL would result in a substrate suitable for the microbial production of added value products as ethanol and PHAs. Although, the LS removal was the smallest of all three, the amount needed to obtained the results presented, its price and manageability make it the best material enhance the bioprocessing of HSSL.

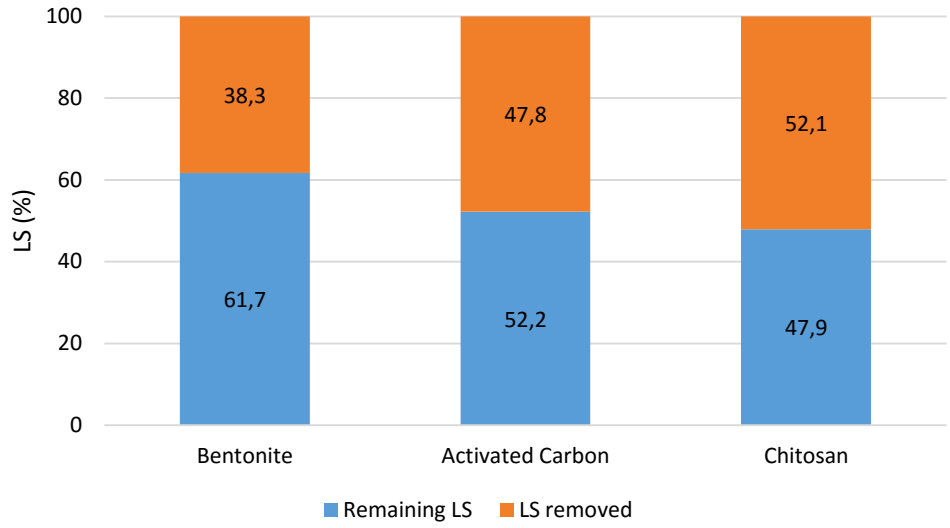

Fig. 7.3. Best LS removal values for each adsorbent tested at pH 2.00. 50, 25 and 70 g L^{-1} for bentonite, activated carbon and chitosan, respectively.

7.3.3. Optimization of bentonite assays

7.3.3.1. Evolution of components of LS by GC-MS analysis

Several compounds were identified in the HSSL before the treatment with bentonite in the control batch (Table 7.4). Among LS, galic acid was the most abundant in HSSL and is usually formed from hydrosable tannis during the sulfite pulping and it has strong antimicrobial properties (Marques et al., 2009). Vanilic acid was also found in HSSL in lower extent than galic acid and it also displays inhibitory effects for microbial growth (Cortez and Roberto, 2010). These compounds are one of the major factors that limits the bioconversion processes using HSSL due to their toxicity (Mussatto and Roberto, 2004, Pereira et al. 2012).

The batches treatment with bentonite performed in the 3.2 section were characterize along with the best assay from 3.1.1., 25 g L^{-1} of bentonite at pH 4.6. Table 7.5 presents the content in weight/weight percentage that are made relatively to the weight extracted originally. This explains that, for example, the amount of galic acid is higher at pH 4.60 and 25.0 g L^{-1}, since other compounds were adsorbed by the bentonite which caused the % (w/w) of the galic acid to rise when compared to the control. The main objective of the results presented is to characterize the HSSL treated with bentonite

Table 7.4. %(w/w) of the assays made with bentonite at lower pH obtained by GC-MS.

Compound	% (w/w) Control	% (w/w) - Bentonite 25 g L^{-1}; pH 2.0	% (w/w) - Bentonite 50 g L^{-1}; pH 2.0	% (w/w) - Bentonite 75 g L^{-1}; pH 2.0	% (w/w) - Bentonite 25 g L^{-1}; pH 4.6
Ethilamine	0.000	0.056	0.000	2.828	0.000
Laevulic acid	2.820	0.118	0.000	0.000	3.511
2-Furancarboxylic acid	10.166	0.360	14.328	20.829	11.074
Succinic acid	3.146	0.137	5.147	11.983	0.000
Malic Acid	1.335	0.055	1.314	1.779	1.812
Isoquinoline	2.154	0.134	1.477	0.000	0.000
Benzaldehyde	7.232	0.294	5.914	8.427	8.124
Phthalic acid	1.464	0.000	0.000	0.000	0.000
2-Methyl-2(p-methoxy)mandelate	0.000	0.067	0.000	0.000	0.000
Vanillic acid	3.234	0.128	3.128	5.948	2.675
3-Vanilpropanol	1.442	0.063	1.221	0.000	0.000
Pyrogalol	0.000	0.691	0.000	0.000	0.000
Syringic acid	16.399	0.055	17.368	29.630	16.466
Gallic acid	48.544	2.390	46.094	37.576	82.996
4H-benzopiran-4-ona	1.988	0.000	0.000	0.000	0.000
Unknow	3.678	0.071	0.000	3.822	2.953
3-Phorbinepropanoic acid	3.218	0.000	2.140	0.000	4.108

in its compositions while also qualitatively comparing the different compounds and their abundance for each individual assay.

The treatment with 25.0 g L^{-1} of bentonite at pH 4.60 left mainly galic, syringic and 2-furanocarboxilic acid. Other compounds such as pthalic acid, isoquinoline and 4H-benzopiran-4-ona were not detected although they were present in the control. It is possible to infer that they were adsorbed by bentonite. Regarding the treatment with 50 g L^{-1} of bentonite at pH 2.00, laevulic acid, isoquinoline, benzaldehyde, phthalic acid were apparently four of the main components adsorb.

It is important to bear in mind that this procedure constitutes a preliminary qualitative analysis of LS fraction present in HSSL. Other components from HSSL were not extracted with the chosen method and an extended characterization should be done in further work.

7.4. Conclusion

The results of this study revealed that adsorbent treatment of HSSL can be a good solution to make it viable and less toxic to bioconversion processes. Although the best removal percentages were obtained using chitosan, bentonite remains the best adsorbent when analyzing the removal yield and its lower price. Chitosan could be used in an industrial level if a partnership with a fish harbor in which crustacean shells could be obtained by a low price making viable its use for HSSL detoxification. Activated carbon seems not to be a good choice due its lack of selectivity towards LS. In all assays performed, pH shows to play a major role in the amount of LS adsorbed as well as the type of agitation. Nevertheless, a careful planning needs to be done to achieve a compromise between the investment in the adsorbent and energy for stirring and the LS quantity needed to be removed.

Further work should include the use of treated HSSL on bioconversion processes and its comparison with the HSSL without adsorbent treatment. Also, the functionalization of the different adsorbents used could be of interest to make them more selective towards LS removal, in addition to the study of applications for the adsorbents with LS to diminish all the process cost.

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Chapter 8

SCOA production through ecoengineering of aerobic sludge

In this chapter, HSSL was used as a substrate to produce SCOA through acidogenic fermentation. SCOA have a broad range of applications in the production of biopolymers, bioenergy and biological removal of nutrients from wastewaters. The acidogenic fermentation took place in a CSTR for 88 days. Selective pressure conditions imposed on the culture were studied by changing parameters as HRT, SRT and temperature. The effect of these parameters on SCOA production was evaluated. The culture required 46 days to adapt to the conditions imposed, reaching a pseudo-steady state after this period. The maximum concentration of SCOA produced, 7.45 gCOD L^{-1}, occurred on the 67^{th} day and corresponded to the highest degree of acidification obtained, 38.0%. Acetic, propionic, butyric, valeric and lactic acids were the SCOA produced throughout the 88 days with an average proportion of 58.6/17.2/19.5/0.8/3.9%, respectively.

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Queirós, D., Sousa, R., Pereira, S., Serafim, L. S.. Short-chain organic acids production through eco-engineering of aerobic sludge.

8.1. Introduction

The increasing interest in new renewable sources of energy and materials is a consequence of several factors, including the rapid depletion of petroleum, and the colossal generation of residues and wastes, both direct consequences of human population growth and its activities. To exploit the potential of using wastes as feedstock, waste management needs to move from treatment-oriented processes to the integration of technologies able to valorize organic waste streams for the production of value-added products (Fava et al., 2015; Lee et al., 2014).

Currently, a considerable effort is being made to develop technologies able to produce value-added products using residues and by-products. Koutinas et al. (2014) reviewed and highlighted several compounds to be produced from wastes. This list included building-blocks as succinic acid, 2,3-butanediol, 1,3-propanediol, biofuels like bioethanol and biohydrogen, polymers such bacterial cellulose and PHAs, and SCOA (Koutinas et al., 2014).

SCOA production is currently achieved by chemical synthesis, followed by distillation at atmospheric pressure (Lee et al., 2014). These chemical compounds have a maximum of six carbon atoms and present a broad range of applications in the production of biopolymers (Frison et al., 2015; Shen et al., 2014), bioenergy (Woo Park et al., 2014) and biological removal of nutrients from wastewaters (Elefsiniotis et al., 2004; Frison et al., 2015).

As mentioned before, industrial and urban wastes can be used for SCOA production, through AF. This is a stage of AnD, where several chemical compounds present in the wastes are transformed into SCOA including acetic, propionic, butyric or lactic acids and alcohols as ethanol (Silva et al., 2013). The AnD is a complex process, which can be divided into four individual stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. If AF is the objective, the process is interrupted at acidogenesis or acetogenesis, inhibiting the methanogenesis. AnD is a mature technology that is usually employed within full-scale facilities worldwide for the treatment of industrial and urban wastewaters and organic solid wastes. AnD is advantageous over aerobic activated sludge systems because of its high organic content removal, low energy input requirements,

energy production, and low sludge production (Ke et al., 2005). Usually, the final goal of AnD is the production of methane and carbon dioxide (biogas), and only recently is being driven by the formation of SCOA. For SCOA production, it is essential to establish selective pressure conditions inside the reactor to inhibit methanogenic microorganisms and select those who are capable of synthesizing SCOA, maximizing their production (Temudo et al., 2009). This selection is possible since methanogenic microorganisms are quite sensitive to reactor conditions as temperature or inhibitors and present low growth rates compared to the acidogenic population (Visvanathan and Abeynayaka, 2012). Furthermore, the thermodynamics of acidogenesis is more favorable than methanogenesis (Aquino and Chernicharo, 2005; Silva et al., 2013). Parameters such pH (Jankowska et al., 2015; Jiang et al., 2013), temperature (Jiang et al., 2013), HRT and SRT (Miron et al., 2000; Scoma et al., 2013) and OLR (Dogan and Demirer, 2009) were already studied in the SCOA production.

AF has, thus, the potential to produce value-added compounds with diverse downstream applications from low-cost waste-based materials. This aspect is of vital importance to the integration of an AF stage into other processes, once the substrate costs often contribute significantly to the overall process economy. Therefore, the critical parameter to be ascertained to select the most suitable waste streams for acidogenic fermentation, and thus to establish a more cost-effective process, include the biochemical acidogenic potential of the waste stream. This means the composition and amount of SCOA that can be generated from the fermentation of the organic constituents (Lee et al., 2014). Sulfite spent liquors have the potential to be valorized through a lignocellulosic biorefinery approach (Rueda et al., 2015). Due to its considerable amount of phenolics and sugars such as glucose, xylose and in less extent galactose, mannose and arabinose, SCOA production is a strong opportunity to be explored. The SCOA generated can be used further in other processes, namely, PHAs production by MMC (Queirós et al., 2016, 2014).

The objective of this chapter was to evaluate the possibility of application of AF to an industrial by-product from the pulp and paper industry, HSSL, to produce SCOA. Instead of using the traditional anaerobic sludge as inoculum of the process, an aerobic

activated sludge was submitted to anaerobic conditions without pH control and temperature controlled at 30 °C. In this way it was expected to easily avoid the presence of methanogenic bacteria, usually strict anaerobes, and increase the presence of the acidogenic population (Visvanathan and Abeynayaka, 2012; Zygmunt and Banel, 2009).

8.2. Materials and Methods

8.2.1. Microbial Culture

The reactor was inoculated with an MMC collected from an aerobic tank of the WWTP Aveiro Sul, SIMRia. The inoculum concentration was 10.5 gVSS L^{-1}.

8.2.2. Experimental Setup

A CSTR configuration was chosen to perform the acidification of chemically pre-treated HSSL under anaerobic conditions. The working volume of the reactor was 1.55 L and the flow rate of the feeding solution was 0.85 L d^{-1} resulting in a HRT of 1.76 days. The reactor had no retention system for the biomass resulting in SRT similar to the HRT. The effluent was collected at the outlet of the reactor by overflow. Reactor stirring was performed by a magnetic stirrer and kept constant at 100 rpm. Nitrogen was sparged regularly to assure anaerobic conditions. Oxidation-reduction potential (ORP) was monitored with a transmitter M300 2-channel, ORP meter (Mettler-Toledo Thornton, Inc). The system worked with temperature control at 30.1 ± 1.0 °C and without pH control.

8.2.3. Substrate

HSSL from magnesium based acidic sulfite pulping of *Eucalyptus globulus* was supplied by Caima – Indústria de Celulose S.A. (Constância, Portugal). Pre-evaporated HSSL was collected from an inlet evaporator of a set of multiple-effect evaporators to avoid the presence of free SO_2. To remove part of the most recalcitrant compounds, HSSL was submitted to a preliminary pretreatment (Pereira et al., 2012). The pretreatment started with a pH adjustment to 7.0 with 6 M KOH, followed by aeration with compressed air (6 hours per liter of HSSL – 6 h L^{-1}). Then, the liquor was centrifuged for 1 h at 5000

rpm. The precipitated colloids were filtered off using a 1 µm glass microfiber filter. Finally, the pre-treated HSSL was stored at 4 °C. The total COD of pretreated HSSL was determined (≈ 267 gCOD L^{-1}). LS and phenolic components were still the main constituents, ca. 190 g L^{-1}, along with xylose, acetic acid and glucose, 43.5, 14.4 and 7.9 g L^{-1}, respectively. No phosphates and ammonia were detected in HSSL.

8.2.4. Fermentation Medium

To achieve an OLR of 11.80 gCOD L^{-1} d^{-1} in the CSTR, HSSL was diluted with a mineral solution (1:12.8). The mineral solution was composed by (per liter of distilled water): 80 mg of $CaSO_4 \cdot 2H_2O$, 160 mg of $FeSO_4 \cdot 7H_2O$, 160 mg of $MgSO_4 \cdot 7H_2O$, 80 mg of $Na_2MoO_4 \cdot 2H_2O$, 160 mg of NH_4Cl.The pH of the medium was adjusted to 7.0 and the medium was autoclaved for 20 min at 121 °C. KH_2PO_4 (160 mg L^{-1}) and K_2HPO_4 (80 mg L^{-1}) were added under sterile conditions.

8.2.5. Sampling

Samples were collected every 4 days, three times a day at intervals of 3 hours (sample volume of 5 mL). The ORP and temperature inside the bioreactor were recorded at the time of each sample collection. Samples were further centrifuged at 13000 rpm for 10 minutes, being the pellet discarded and the pH of the supernatant measured before storage under -16 °C for later determination of glucose, xylose, SCOA, COD and LS concentrations. 5 mL samples were regularly collected for determination of TSS and VSS.

8.2.6. Analytical Methods

Biomass concentration was determined using total suspended solids (TSS) and volatile suspended solids (VSS) procedure described in *Standard Methods* (Clesceri et al., 1998).

COD was measured accordingly to *Standard Methods* (Clesceri et al., 1998).

Acetic, propionic, butyric, valeric and lactic acids, xylose and glucose were measured by HPLC. 650 µL of each sample were filtered using centrifuge tube filters with a cellulose acetate membrane, 0.2 µm pore size at 8000 rpm for 20 minutes. Samples

were then injected (Auto-sampler HITACHI L-2200) in an ion exchange column Aminex HPX-87H at 40 °C (Oven Gecko-2000, CIL CLUZEAU), and analyzed by a refractive index detector (HITACHI L-2490). The eluent 0.01 N H_2SO_4 was pumped at a flow rate of 0.6 mL min^{-1} (HITACHI L-2130 pump) at room temperature. The eluent was prepared with milli-Q water and filtered with a cellulose acetate membrane, 0.22 µm pore size. The concentrations of sugars and SCOA in g L^{-1} were determined by comparison with the calibration curves of each analyzed compound obtained using standards of known concentrations. The standards concentrations were within the range of the expected concentrations of the analytes: 0.15 g L^{-1} to 3.00 g L^{-1} for lactic and valeric acids; 0.20 g L^{-1} to 4.00 g L^{-1} for propionic and butyric acids; 0.25 g L^{-1} to 5.00 g L^{-1} for glucose, xylose and acetic acid.

LS were measured according to Restolho et al. (2009) The absorbance of samples was measured in a Spectrophotometer (Shimadzu UVmini-1240) at 273 nm, after a dilution of 1:400. LS concentration was calculated using the Beer-Lambert law with a molar attenuation coefficient of 7.41 g^{-1} cm^{-1} (Xavier et al., 2010).

8.2.7. Calculations

The values of SCOA, sugars and biomass in g L^{-1} were converted in gCOD L^{-1} using conversion factors that represent the mass (g) of oxygen required to oxidize 1 g of compound based on the oxidation reactions for each compound. The overall oxidation equation is represented below.

$$\text{a compound} + \text{b}\ O_2 \rightarrow c\ CO_2 + d\ H_2O + e\ NH_3 \qquad (8.1)$$

In which *a*, *b*, *c*, *d* and *e* represent the stoichiometric coefficients of the equation. Therefore, the conversion factor (cf) was calculated according with the following equation:

$$cf\ (gO_2/g) = \frac{b \times M(O_2)}{a \times M(compound)} \qquad (8.2)$$

The conversion factors were 1.07 gO_2 g^{-1} for glucose, xylose, lactic and acetic acids, 1.51 gO_2 g^{-1} for propionic acid, 1.82 gO_2 g^{-1} for butyric acid, and 2.04 gO_2 g^{-1} for valeric acid. For biomass, it was assumed an empirical molecular formula of $C_5H_7NO_2$ that corresponded to a conversion factor of 1.42 gO_2 g^{-1} for biomass (Queirós et al., 2014).

Also, acidification degrees (AD) were calculated for the fermentative process. The total acidification degree (AD_{Total}) represents the amount of substrate consumed to produce SCOA considering all the organic matter entering the reactor (Equation 8.3). The sugars acidification degree (AD_{Sugars}) represents the amount of sugars consumed to produce SCOA considering the xylose and glucose fed to the reactor (Equation 8.4). These calculations were performed as percentages.

$$AD_{Total}\ (gCOD/gCOD) = \frac{[SCOA]}{COD_{In}} \times 100 \qquad (8.3)$$

$$AD_{Sugars}\ (gCOD/gCOD) = \frac{[SCOA]}{COD_{Sugars}} \times 100 \qquad (8.4)$$

For the effluent, the yield on SCOA was calculated relatively to fed COD, represented by $Y_{SCOA/S}$ (Equation 8.5) and relatively to consumed sugars (xylose and glucose), $Y_{SCOA/Sugars}$ (Equation 8.6).

$$Y_{SCOA/S}\ (gCOD/gCOD) = \frac{[SCOA]_{produced}}{(COD_{In} - [SCOA]_{in}) - (COD_{out} - [SCOA]_{out})} \qquad (8.5)$$

$$Y_{SCOA/Sugars}\ (gCOD/gCOD) = \frac{[SCOA]_{produced}}{COD_{Sugars\ in} - COD_{Sugars\ out}} \qquad (8.6)$$

The substrate consumption volumetric rate ($-r_s$) and the SCOA production volumetric rate (r_p) in gCOD L^{-1} h were also calculated by dividing the substrate consumed or product formed, respectively, by the HRT.

8.3. Results and Discussion

A CSTR working under AF conditions usually requires the imposition of a selective pressure to promote the presence of acidogenic bacteria and decrease the amount of methanogenic bacteria that could deviate carbon source towards methane production instead of SCOA (Visvanathan and Abeynayaka, 2012). In the present work, an aerobic inoculum was used and no selective pressure to avoid methanogens was imposed. The choice of an aerobic inoculum was based on the fact that most methanogens are strict anaerobes and most of the acidogenic bacteria are facultative anaerobes (Fernández-Morales et al., 2010; Visvanathan and Abeynayaka, 2012). In this way, the inoculum was expected to be poor in methanogens and the anaerobic conditions of the CSTR would work as a selective pressure to wash out the strict aerobes that would not contribute to the AF process (Fernández-Morales et al., 2010). Due to the possibility of a marked decrease in biomass concentration, 1.3 L of inoculum, near the reactor working volume, corresponding to a concentration of 10.5 gVSS L^{-1} were used.

The CSTR was operated with a HRT of 1.76 days. Longer HRT are usually advantageous to AF since microorganisms have more time adapt to the waste used (Lee et al., 2014). However, as Fang and Yu, (2000) verified, successive increases in HRT will not lead to a higher organic matter conversion into SCOA, reaching a plateau phase. Also, to achieve such HRTs, larger reactors are required which contribute to the process costs. On the other hand, shorter SRTs were observed to promote the growth of acidogenic organisms comparatively to methanogens, usually presenting low growth rates (Lee et al., 2014). For this reason, it was chosen not to increase the HRT.

The initial COD concentration of the CSTR feed, 20.8 g L^{-1}, was chosen considering preliminary assays of HSSL acidification (data not shown) and considering COD concentration of HSSL after chemical pre-treatment, 266 gCOD L^{-1} with a significant high content of LS, 190 g L^{-1}. Temperature was held in the mesophilic range, 30 °C, since at this value it is considered that the process occurs efficiently without major energy requirements (Lee et al., 2014). Also, preliminary tests showed an improvement of SCOA production at this temperature than without temperature control (data not shown). Finally, pH was not controlled but monitored along the CSTR operational period. This can

be advantageous at industrial level considering that lower amounts of chemicals are required and no extra equipment for pH control needed, thus reducing operation costs. This would also be beneficial from the scale up of the process point of view.

8.3.1. Acidogenic Fermentation of HSSL

The variation of SCOA composition during the CSTR operational period, that lasted about 88 days, as well as of the main sugars, glucose and xylose, is shown in Fig. 8.1.

Glucose was consumed by the culture preferentially to xylose, which only started to be consumed after the depletion of the former. This agreed with sugars metabolism, since the metabolic pathway for glucose conversion is simpler than for xylose. Glucose enters directly in glycolysis step producing pyruvic acid, from which SCOA are synthetized (Temudo et al., 2009). On the other hand, xylose needs to be converted to the intermediary D-xylulose-5-phosphate, and go through pentose phosphate pathway (most commonly) to finally arrive in glycolysis and then converted to pyruvate and consequently into SCOA (Jeffries, 1983; Prakasham et al., 2009). Despite the catabolic ATP yield per carbon mole of substrate being higher for xylose, the consumption of pentoses is not so advantageous for the culture since it is not so readily converted as glucose (Temudo et al., 2009).

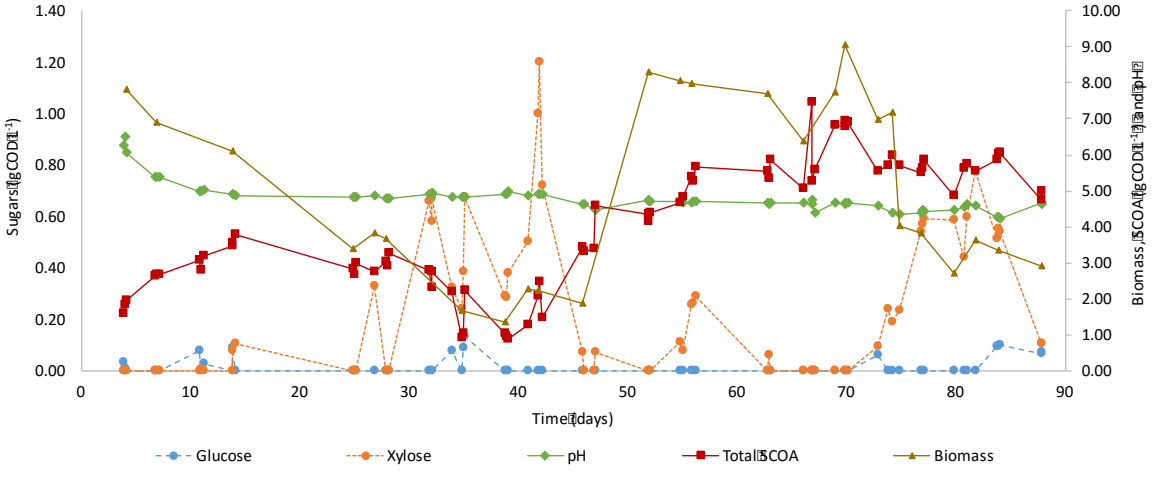

Fig. 8.1. Evolution of SCOA, sugars, biomass and pH variation during the fermentative process.

During the first 15 days of operation, there was a gradual increase of SCOA produced, stabilizing at day 30. Between days 30 and 40, a drastic decrease in SCOA production that resulted in an increase in sugars concentration (glucose and xylose) in the reactor was observed. The minimum value of total SCOA concentration, 0.86 gCOD L^{-1}, was obtained on day 39 (Fig. 8.1 and 8.2). This could be a consequence of the lowest biomass concentration observed on this day in the CSTR (Fig. 8.1), suggesting that the culture was still unstable. The pH also fluctuated due to the instability verified once SCOA were produced in a lower concentration in the reactor.

Total SCOA maximum concentration achieved was 7.45 gCOD L^{-1} on day 67, which was comparable to the values reported in the literature. For instance, Bengtsson et al. (2008) performed an AF study in batch with pH control at 6.0 in which four industrial wastes were tested. In this study, the maximum SCOA concentration obtained was 3.96 gCOD L^{-1} for a waste derived from a paper mill. Additionally, Silva et al. (2013) also tested the acidification of eight organic streams in batch experiments and the maximum SCOA concentration obtained was 3.37 gCOD L^{-1} for cheese whey. Lastly, Jie et al. (2014) studied different pH values and their impact on AF of excess sludge and verified that a higher SCOA production was obtained for pH 10.0, corresponding to a concentration of 3.16 gCOD L^{-1}. Noticeably, the SCOA concentration obtained was related not only to the conditions imposed during the AF process but also to the acidification potential of the waste used. In this way, HSSL is a complex and rich substrate in not only toxic components but in sugars possible to be fermented into SCOA.

Sugars consumption and SCOA production were chosen as the parameters to evaluate the stability of the microbial population. A more stable substrate consumption and SCOA production, 5.50 ± 0.84 gCOD L^{-1}, were obtained from day 45 onwards and a pseudo-stationary state was then reached meaning that, after inoculation, the MMC required 44 days to adapt to substrate and conditions imposed (Table 8.1). Considering the results obtained by Bengtsson et al. (2008), using two pulp and paper mill effluents as substrate in batch experiments, in which the stabilization occurred in day 17, the 45 days needed with HSSL correspond to a significant longer adaptation period.

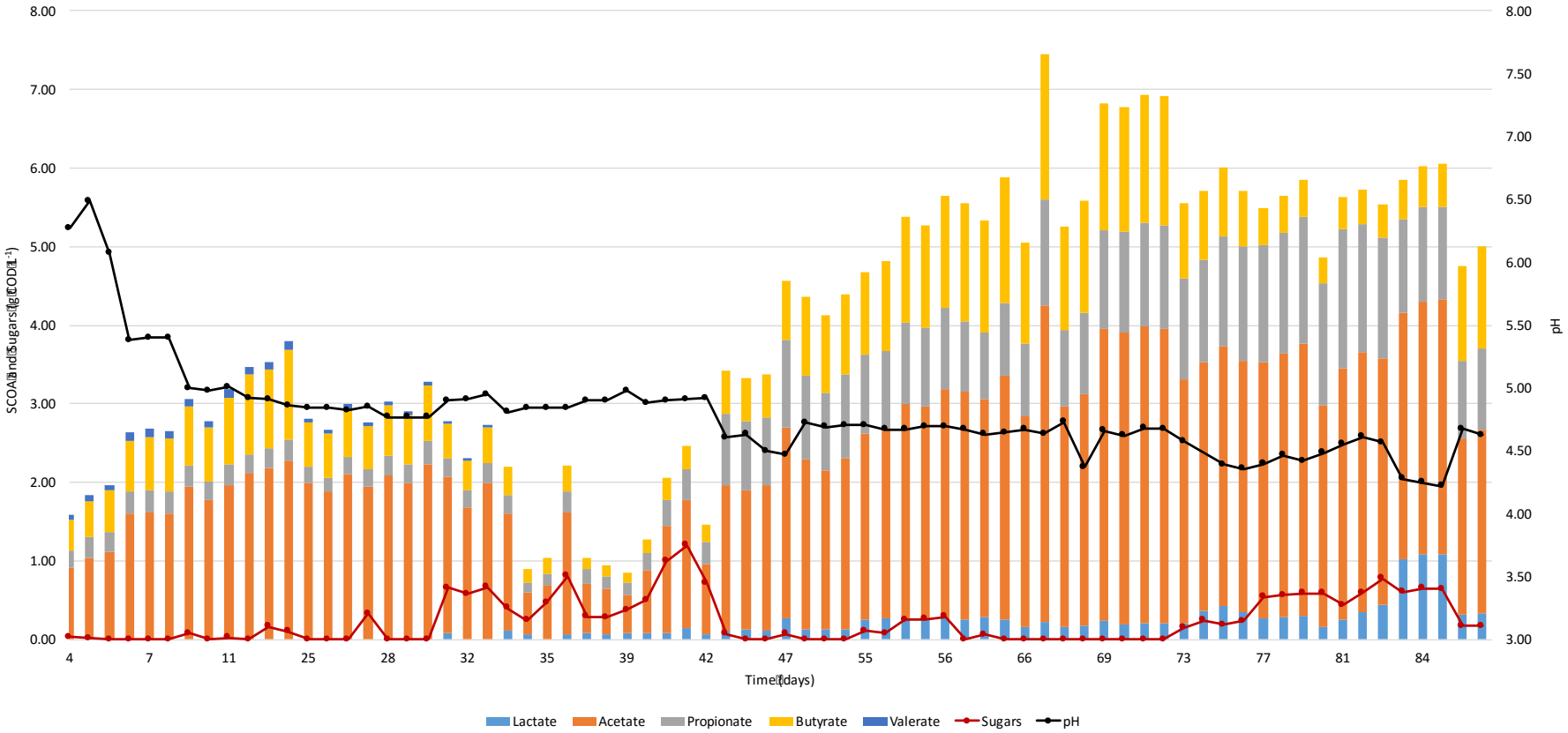

Fig. 8.2. SCOA production profile, sugars and pH evolution throughout fermentation time.

Table 8.1. Main results from acidogenic fermentation of HSSL.

	Time	HRT/SRT	OLR	Sugars consumed*		SCOA*	SCOA profile*					AD*
				gCOD L^{-1}	(%)		Lactic acid	Acetic acid	Propionic acid	Butyric acid	Valeric acid	
Operation	0 – 88	1.76	11.8	3.77±0.29	94.0±7.23	4.00±1.76	3.91± 3.91	58.6± 6.84	17.2± 6.80	19.5± 6.49	0.76± 1.26	20.4±8.98
PSS	46 – 88	1.76	11.8	3.78±0.25	94.4±6.28	5.50±0.84	5.71± 3.91	53.6± 3.21	22.0± 4.21	18.7± 7.49	0.00± 0.00	28.1±4.26

*(mean values ± standard deviation)

Units: Time - days; HRT/SRT – days; OLR - gCOD L^{-1} d^{-1}; SCOA - gCOD L^{-1}; AD - %SCOA profile - %

Such long adaptation period to reach a steady state could be explained due to the high content of microbial inhibitors in HSSL. As reviewed by Pereira et al. (2013), HSSL contains compounds such as gallic acid, pyrogallol and furfural, known bacterial inhibitors and, consequently, could affect the production of SCOA and biomass growth. An introduction of a pre-adaptation step in batch mode could accelerate the adaptation of MMC to the media.

Finally, the MMC used might need some extra time of adaptation given not only the presence of recalcitrant compounds like LS and many highly toxic phenolic compounds but also due to the loss of obligate aerobes in the first days of the process (Fernández-Morales et al., 2010; Pereira et al., 2013)

8.3.2. SCOA Profile

As stated previously, pH was not controlled during the process and its variation is shown in Fig. 8.2. The initial value of pH in the CSTR was 6.27, which could be related to pH of HSSL. In the following days, a decrease of pH was observed until day 8, when a stabilization occurred around 4.85 until day 42. Then pH suffered a new decline to an average value of 4.7 until the end of the operational period, although lower values of pH, around 4.5, were observed between 75 and 85 days. The decrease in pH value during the process was related to the increase in SCOA concentration in the reactor. However, this parameter remained quite stable during the operational period probably due to the buffer capacity of HSSL, as previously observed by some authors. The buffer capacity of HSSL combined with the use of a CSTR prevented SCOA accumulation in the medium and pH reduction to inhibitory values.

It is important to highlight the fact that the period during which SCOA production was maximum, corresponded to pH values lower than 5.00. Previous studies indicated that SCOA production, in general, results in very low yields in this range of pH values (Bengtsson et al., 2008; Lee et al., 2014). The results obtained are probably related to the use of aerobic cultures usually less sensitive than anaerobic, thus tolerating extreme conditions easily. This is an advantage of this process since, in this pH range, methanogenic microorganisms cannot survive (Gerardi, 2003). Low pH values recorded

proved to be an effective way to improve SCOA formation without the need to add inhibitors for methanogenic bacteria and without affecting the yield of the process.

Acetic, propionic and butyric acids were the SCOA produced in higher quantities during the operational period, which was already expected since these are usually the main products from this bioprocess (Lee et al., 2014). During PSS, 2.96 ± 0.53 gCOD L^{-1} of acetic, 1.20 ± 0.24 gCOD L^{-1} of propionic and 1.03 ± 0.45 gCOD L^{-1} of butyric acids were produced. Acetic acid was, most of the time, the main product of the fermentation of HSSL, followed by propionic acid (Fig. 8.2). The low pH values during the operation seemed to favor acetic acid production, which was the opposite to what Jiang et al. (2013) observed, that at higher pH values butyric acid was favored to acetic acid. Also, the temperature at which the bioprocess was carried out, 30 °C , could also enhance the production of acetic acid (Pittmann and Steinmetz, 2014). Lastly, Chang et al. (2010) reviewed the AF of several substrates, such as food wastes, pig and chicken manure, rice straw and corn stover and in almost all assays, acetic acid was the main SCOA produced besides propionic and butyric acids.

Propionic and butyric acids concentrations appeared to be somehow inversely related, since their composition changed symmetrically. This fact is in agreement with the literature that reports different metabolic pathways for the production of the two acids, suggesting the existence of two different types of population competing for the carbon source (Bengtsson et al., 2008; Cohen et al., 1984; Horiuchi et al., 2002).

SCOA concentrations in the effluent were also studied during PSS, and it contained 2.36 gCOD L^{-1} of acetic, 0.87 gCOD L^{-1} of propionic, 0.83 gCOD L^{-1} of butyric and 0.18 gCOD L^{-1} of lactic acids, resulting in a total SCOA concentration of 4.24 gCOD L^{-1}.

SCOA composition changed with pH. At the beginning of the CSTR operation, on day 4, the percentages of acetic, propionic, butyric, valeric and lactic acids were 57/14/25/4/0%, respectively, corresponding to a maximum pH value of 6.27. At day 84, the lowest pH value of 4.22 was achieved, corresponding to a SCOA profile of 54/20/9/0/18%, which prove the influence of pH on the SCOA distribution. During the first 32 days, valeric acid was also produced up to a maximum value of 0.107 gCOD L^{-1}. Experimental data showed that valeric acid production could be associated with the

higher pH values at which the system was operated at the beginning of the process. Thus, as can be seen (Fig. 8.2), the concentration of valeric acid decreased with the decrease of pH, caused by the increase in SCOA production in the system. This fact is consistent with the results obtained by Lim et al. (2008) for food waste acidification after testing three pH values in a semi-continuous reactor (once-a-day feeding and draw-off), 5, 5.5, and 6, and observed that at pH 5 valeric acid was not produced (Lim et al., 2008). Regarding lactic acid, the results showed a relation with pH which is in agreement with some studies that stated that lower pH values or periods of reactor instability favor lactate production (Gouveia et al., 2016; Itoh et al., 2012; Wu et al., 2015). Thus, lactic acid started to be produced when pH values dropped below 4.92, and achieved a maximum concentration at pH 4.25 after 84 days of fermentation. The maximum lactic acid obtained corresponded to 18% of the total SCOA (Fig. 8.2). The pH proved to be a crucial parameter in the definition of the SCOA profile during AF. This could be a consequence of the pH influence on the selection of the MMC and its ability to produce different SCOA since different bacterial populations could produce different SCOA (Jie et al., 2014; Lee et al., 2014). Moreover, a tight relationship between pH and type of SCOA was not yet established. Such inference, so far, should only be done when working with the same kind of residue/waste used (Lee et al., 2014). Monitoring the distribution of the pH and SCOA produced along the process is essential in this type of process since different types of SCOA mixtures could be applied in various bioprocesses, as mentioned earlier.

Besides the control of SCOA profile, along with the temperature, pH also affects the solubility of organic matter, by influencing the hydrolysis step, which is essential to guarantee the conversion of all components. In the particular case of HSSL, the hydrolysis step is not critical since the sulfite process led to nearly complete hydrolysis of hemicelluloses and lignin (Pereira et al., 2013; Xavier et al., 2010). In this way, a lot of monomeric sugars are already available to be converted to SCOA through AF (Pereira et al., 2013; Xavier et al., 2010).

8.3.3. Biomass profile

In the beginning, the CSTR lost a significant amount of biomass due to the adaptation of MMC to the medium, reaching 1.33 gCOD L^{-1} at day 39, as can be seen in Fig. 8.1. Since an aerobic MMC was used, only facultative microorganisms were able to survive and all obligate aerobic together with anaerobes that consumed carbon slowly ended up being wash out from the reactor in the effluent stream (Fernández-Morales et al., 2010). Then, between days 39 and 41, a small increase in biomass to 2.27 gCOD L^{-1} was observed, to be followed by a decrease until the 46^{th} day. A quick increase in the following five days took place, which was coincident with the entrance in the pseudo-stationary state and was kept constant until day 70. During PSS, a new decrease in biomass concentration was coincident with an increase of sugar in the medium, probably due to a small instability of the system that did not interfere with the SCOA production. The same pattern was observed by Fernández-Morales et al. (2010) during the acclimatization of conventional aerobic activated sludge to obtain an enriched acidogenic culture. Authors theorized that such evolution could be explained by the presence of three types of microorganisms, facultative, acidogenic and strict aerobic microorganisms, in the conventional activated sludge inocula. Facultative microorganisms were able to grow during the first period of operation, while strict aerobes were being slowly removed from the systems (Fernández-Morales et al., 2010). Acidogenic microorganisms gradually became the dominant group as the pH of medium decreased, inhibiting facultative ones. Since this fraction in the inoculum represents a small portion of the bacteria and due to the high toxicity of HSSL, a considerable amount of time (46 days) was required to reach a stable operation (Fernández-Morales et al., 2010; Pereira et al., 2013).

8.3.4. Acidification Degree

Acidification degrees (AD) relatively to the total COD, AD_{total}, and to the main sugars (xylose and glucose), AD_{sugars}, were calculated for all samples taken. AD_{total} had a maximum of 38.0% on day 67, which corresponded to the maximum value of SCOA concentration. The reason for the relatively low AD_{total} can be explained by most the COD

present in the feed being constituted by phenolic compounds, LS and other recalcitrant compounds, which are difficult to biodegrade with the retention times applied.

The maximum AD_{sugars} obtained was 185.9% at day 67. This value shows that not only xylose and glucose but other compounds present in the HSSL were consumed. These compounds include other monomeric sugars such as rhamnose, arabinose, mannose or galactose (Pereira et al., 2013), bellow the detection limit of HPLC due to the dilution applied, were consumed. Assuming the data provided by Caima – Indústria de Celulose S.A., HSSL after chemical pre-treatment would have 1.36, 1.14, 2.28, 2.28 gCOD L^{-1} of rhamnose, arabinose, mannose and galactose, respectively. Taking these values into consideration and its total consumption, the $AD_{TotalSugars}$ would be 163.5%. This means not only sugars were acidified, but the culture also had the capacity to metabolize and convert some phenolic components or even LS. Hence, it would be interesting to make a more detailed evaluation of HSSL components to understand which compounds were being consumed to produce SCOA.

Moreover, higher HRT values should be imposed to the CSTR to assess whether the most recalcitrant compounds of HSSL, LS and phenolics, could be fermented, since at a HRT of 1.76 days its consumption occurred at a very small extension. The possibility of maximizing the number of components of HSSL acidified, not only increases the concentration of SCOA obtained but also decreases the amount of possible microbial inhibitors, which favors the utilization of the fermented stream in other bioprocesses.

8.4. Conclusion

Acidogenic fermentation of HSSL was conducted along 88 days in a CSTR. The MMC took about 45 days to adapt to the imposed conditions and reach a pseudo-steady state. At day 67 of operation, a maximum of SCOA concentration was obtained, 7.45 gCOD L^{-1}, that corresponded to an AD of 38.0%. The results obtained in this work proved that HSSL is a potential candidate to be used as substrate for an AF. The choice of using an aerobic culture and leaving the pH uncontrolled proved to be a decision that led to a significant SCOA production. In a future scale-up of the process, this could mean a reduction in the process cost. Further studies should address the consequences of using higher HRT,

different reactor conformation (e.g. moving bed biofilm reactor) and deepen the knowledge of how and which carbon compounds in the HSSL are being utilized. These changes will allow maximizing the SCOA production for further applications.

8.5. References

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Chapter 9

Assessing the influence of pH and reactor configuration on acidification of HSSL components

Technologies to convert renewable carbon into the selected building blocks are a relatively unexplored sector, with low efficiencies/yields when compared to petrochemical industry. Lignocellulosic biomass, due to its abundance, low-cost and broad availability, presents itself as a promising substrate to produce chemicals and energy. SCOA present a great potential not only due to their broad applicability but also to the fact that can be produced biologically through AF from renewable resources, such HSSL. The optimization and control of this process are crucial, and two relevant parameters stand out pH and reactor configuration.

In the present study, the AF of HSSL was evaluated in CSTR, without (CSTR-wo/pH) and with pH control (CSTRpH). For CSTR-wo/pH, two retention times were tested, 2.34 and 3 days, being the average SCOA concentrations achieved 3.10 and 3.53 gCOD L^{-1}, respectively. For CSTRpH, three pH values were tested, pH 6, 7 and 8. The average SCOA concentrations obtained were 2.36, 2.38 and 2.27 gCOD L^{-1}, respectively. Lastly, an MBBR was also tested and an average concentration of 2.71 gCOD L^{-1} of SCOA was obtained. The main products were acetic, propionic, butyric and, possibly, succinic acid.

9.1. Introduction

In the bioeconomy age, fermentative production of chemicals and biopolymers should rely on the utilization of renewable carbon sources. Efforts should be made to move from a linear economy ("cradle-to-grave" production system) to circular economy, where wastes gain new value by being used as feedstocks to produce other value-added compounds.

Bio-based processes will allow producing platform chemicals and intermediates that could replace basic petrochemicals and their precursors. However, technologies to convert renewable carbon into the selected building blocks are a relatively unexplored sector, with low efficiencies/yields when compared to petrochemical industry (Koutinas et al., 2014). Nevertheless, U.S. Department of Energy highlighted a set of value-added chemicals to be produced from biomass (Werpy and Petersen, 2004). These building blocks could be either already generated by the petrochemical industry or constitute novel products derived from biomass being produced from renewable carbon through green chemical conversion routes or microbial conversions.

SCOA are aliphatic monocarboxylic acids, with a vast number of applications in food, beverages, pharmaceutical and chemical industries as building-blocks. Also, they have an important role as intermediates in many biological processes (Lee et al., 2014).

SCOA can be produced through AF of wastes, a step from a worldwide spread technology, called anaerobic digestion. Several operational parameters have a direct influence on the AF process: over conversion rates, acidifications degrees and yields of products on substrate. A particular attention should be given to the waste used since it should be rich in organic matter with COD greater than 4000 mg L^{-1} (Lee et al., 2014), ammonium content lower than 5000 mg L^{-1} to avoid inhibition of SCOA production and its availability and the amount of waste generated have to be taken into consideration to ensure stable and continuous waste supply for the production of SCOA (Salehizadeh and Van Loosdrecht, 2004).

HRT and SRT should be planned to allow enough time to organisms to adapt and react with the substrate as well as to inhibit the growth of methanogenic microorganisms (Lee et al., 2014). Regarding the OLR, a linear dependence range can usually be observed

while increasing the amount of substrate fed to the bioreactor until an optimum value (Yu, 2002). After that, the performance of acidogenic population decreases probably due to the rheology of medium and the associated mass transfer acute changes or by the unbearable toxicity of the substrate used (Rincón et al., 2008).

The reactor configuration and the pH are other two important parameters, being the former tightly related to the applied HRT, SRT or OLR. The pH applied to the reactor is critical to produce SCOA because most of the acidogens cannot survive in extremely acidic (pH 3) or alkaline (pH 12) environments. However, specific ranges of pH depend directly on the type of substrate, once it can determine the availability of soluble substrates for the culture (Lee et al., 2014). Besides, pH can also affect the type of SCOA produced from acidogenic fermentation, mainly acetic, propionic, and butyric acids. By manipulating this parameter, it is possible to obtain different SCOA profiles, which are crucial factor when further bioprocesses are dependent on the SCOA profile of the effluent collected. As an example, the SCOA profile fed is directly connected with the composition of PHA produced by MMC (Albuquerque et al., 2011; Duque et al., 2014).

The reactor configuration chosen must take into account the requirement of less capital, less area, less necessity of operation and also should be the most reliable and efficient choice when compared to other well-established options (Lee et al., 2014; Ozgun et al., 2013). Thus, the system should be able to support high OLR and HRT with the minimum operation and maintenance requirements. Two common technologies used in the AF production of SCOA from waste are attached growth and suspended growth.

Among the configurations that use suspended growth, CSTR involves complete mixing of waste and biomass. This can be approximately achieved by well-designed impellers, baffles and reactor shape. An associated challenge is to avoid using too high agitation that can result in damage to the suspended microbes by the shear stress. When designed and operated correctly, a CSTR is ideal to mix waste and microbes thoroughly the presence of suspended solids in the waste. Also, in most CSTR systems, the HRT equals the SRT, and the biomass that did not adapt to the substrate and the excess are removed in the effluent. The HRT used must be longer enough for the microorganisms to adapt, thus preventing washout and the failure of the process. Furthermore, there are

some cases in which CSTR are coupled with a system of biomass recirculation to avoid washout, and in this cases, SRT is higher than HRT (Ozgun et al., 2013). One way to retain biomass inside the reactor is by using mobile supports in which biomass can attach and grow, forming a biofilm in the surface of these supports. In moving bed biofilm reactors (MBBR), biomass can be effectively retained by attaching to carrier materials, which are in constant movement and dispersed through the system. The carriers provide a higher surface for growth of the attached microorganisms, and for the conversion of the usually complex substrates. MBBR incorporates the best characteristics of processes with growth of biomass in suspension and adhered biomass (biofilm) and can be used with high OLR (Karadag et al., 2015; Oliveira et al., 2014).

In chapter 8 was proven to be possible to establish an acidogenic fermentation of HSSL with an aerobic culture as an inoculum and without pH control. The objective of this study was, then, to assess the influence of other operational parameters, namely pH, in the SCOA profile produced, and reactor configuration on the acidification of HSSL.

9.2. Materials and Methods

9.2.1. Culture

The MMC used in this work was collected from an aerobic tank of the WWTP Aveiro Sul, SIMRia. The aerobic MMC was used to inoculate the CSTR with pH control and the MBBR. An acidogenic culture was retrieved from a working CSTR (chapter 8) to inoculate the CSTR without pH control. The biomass concentration in the reactor and in the WWTP sludge was determined by analysis of TSS and VSS, according to *Standard Methods* (Clesceri et al., 1999).

9.2.2. Substrate

The HSSL from magnesium based acidic sulfite pulping of *Eucalyptus globulus* was provided by Caima – Indústria de Celulose S.A. (Constância, Portugal). The pre-evaporated HSSL was collected from an inlet evaporator of a set of multiple-effect evaporators to avoid the presence of free SO_2. Before HSSL utilization part of its most recalcitrant compounds were removed using a preliminary pretreatment (Pereira et al., 2012) which

started with a pH adjustment to 7.0 with 6 M KOH, followed by aeration with compressed air (6 hours per liter of HSSL, 6 h L^{-1}). Then, HSSL was centrifuged for 1 h at 5000 rpm and the precipitated colloids were filtered off using a 1.2 µm glass microfiber filter. The total COD of the pretreated HSSL was determined (≈ 259 gCOD L^{-1}), being the LS the main components (≈ 175 g L^{-1}) along with xylose, acetic acid and glucose (40.8, 13.7 and 6.4 g L^{-1}, respectively). Finally, the pretreated HSSL was stored at 4 °C.

9.2.3. Fermentation Medium

The fermentation medium used was composed of nutrients and the pretreated HSSL. To achieve an OLR of 7.62 gCOD L^{-1} d^{-1} (HRT = 2.34 days) firstly and then of 5.95 gCOD L^{-1} d^{-1} (HRT = 3 days) in the reactor, HSSL was diluted with a mineral solution (1:12.8). The adjustment of HRT from 2.34 to 3 days was carried out only in the CSTR without pH control (CSTR-wo/pH) at day 181. Thus, both the CSTR with pH control (CSTRpH) and MBBR were operated at an HRT of 3 days and with an OLR of 5.95 gCOD L^{-1} d^{-1}. The fermentation medium was composed by (per liter of distilled water): 80 mg of $CaSO_4 \cdot 2H_2O$, 160 mg of $FeSO_4 \cdot 7H_2O$, 160 mg of $MgSO_4 \cdot 7H_2O$, 80 mg of $Na_2MoO_4 \cdot 2H_2O$, 160 mg of NH_4Cl. A solution with 160 mg L^{-1} of KH_2PO_4 and 80 mg L^{-1} K_2HPO_4 was prepared separately to avoid precipitation with the magnesium salts during sterilization. The pH of the medium was adjusted to 6.0 with 6 M KOH and the two solutions were autoclaved for 20 min at 121 °C. Phosphates were added to the feed under sterile conditions and at room temperature.

9.2.4. Experimental Setup

9.2.4.1. Continuous Stirred Tank Reactors

CSTR configuration was chosen to accomplish the AF of the pretreated HSSL under anaerobic conditions. Two independent CSTR were operated, one without pH control, CSTR-wo/pH, and the other with pH control, CSTRpH, at 6.0 ± 0.1, 7.0 ± 0.1 and 8.0 ± 0.1, by the addition of 2 M NaOH and 1 M HCl. The working volume of both CSTRs was 2 L. For CSTR-wo/pH an HRT of 2.34 and 3 days were imposed, and CSTRpH was always operated always with an HRT of 3 days. Since the reactors worked in continuous mode and had no

system for retaining the biomass, the SRT was the same as the HRT. Reactor stirring was performed by a magnetic stirrer and kept constant at 100 rpm. Furthermore, nitrogen was sparged regularly to assure anaerobic conditions. Both CSTR systems worked with temperature control at 30.0 ± 1.0 °C.

9.2.4.2. Moving Bed Biofilm Reactor

MBBR conformation was also chosen to accomplish the acidogenic fermentation of HSSL. The HRT imposed was also of 3 days. The reactor was filled with 41% (1.5 L) cylindrical polyethylene carriers Bioflow 9, supplied by RVT Process Equipment GmbH. Bioflow 9 carriers present as technical properties a packing density of 145 kg $(m^3)^{-1}$, a specific surface area of 800 m^2 $(m^3)^{-1}$ and a dimension of 9 x 7 mm (diameter x height). The mixing of the MBBR was constant and carried out by a submerged pump fixed on the bottom of the reactor. Since the reactor worked with a system for retaining biomass, the SRT was higher than HRT. The effluent was collected at the outlet of the reactor by overflow. Furthermore, nitrogen was sparged regularly to assure anaerobic conditions. The jacketed MBBR system worked with temperature control at 30.0 ± 1.0 °C. Lastly, the initial activated sludge concentration in the reactor was 11.6 g L^{-1}.

9.2.5. Sampling

Samples of 5 mL were collected every day from the reactors, two times a day. Samples were centrifuged at 13000 rpm for 15 minutes. Then, the pellet was discarded and the supernatant was stored in the freezer under -16 °C for further determination of glucose, xylose, SCOA, COD and LS concentrations. Moreover, 5 mL samples were collected every day for TSS and VSS determination.

9.2.6. Analytical Methods

COD was measured accordingly to *Standard Methods* (Clesceri et al., 1999). After properly dilution of samples accordingly with the detection range of the method, the absorbance was read with a colorimeter, Spectroquant Picco COD/CSB (Merck Millipore).

HPLC was used to determine the concentration of SCOA in the collected samples. Samples were filtered with 0.2 µm pore size filters. Then, 20 µL of sample were injected (Auto-sampler Hitachi L-2200) in an anion exchange column (Rezex™ ROA-Organic Acid H+ (8%)) connected to a refraction index detector (Hitachi RI L-2490). The temperature of column was set at 65 °C in a Gecko 2000 external oven and the eluent used was 0.05 N H_2SO_4, prepared with Milli-Q water. The eluent had a flow rate of 0.5 mL min^{-1} (Hitachi L-2130).

The determination of the content in LS of the samples was carried out in accordance to Restolho et al. (2009). The samples were diluted 1:200 and their absorbance was measured in a UV spectrophotometer at 275 nm. The LS concentration was calculated based on the Beer-Lambert law, with a molar extinction coefficient of 7.41 g^{-1} cm^{-1}.

Biomass concentration was determined using total suspended solids (TSS) and volatile suspended solids (VSS) procedure described in Standard Methods (Clesceri et al., 1999).

9.2.7. Calculations

The HRT was calculated by the division of the reactor working volume for the flow rate of the pump (Equation 9.1).

$$\mathrm{HRT\ (days)} = \frac{\mathrm{Workingvolumeofreactor(L)}}{\mathrm{Pumpflowrate(L/d)}} \qquad (9.1)$$

The OLR was determined by the Equation 9.2.

$$\mathrm{OLR\ (gCOD/L\ d)} = \frac{\mathrm{COD_{Feed}}}{\mathrm{HRT}} \qquad (9.2)$$

The values of SCOA, xylose, glucose and biomass in g L^{-1} were converted in gCOD L^{-1} using conversion factors that represent the mass (g) of oxygen required to oxidize 1 g of compound based on the oxidation reactions for each compound. The overall oxidation equation is:

$$\text{a compound} + \text{b}\ O_2 \rightarrow c\ CO_2 + d\ H_2O + e\ NH_3 \qquad (9.3)$$

In which *a*, *b*, *c*, *d* and *e* represent the stoichiometric coefficients of the equation. Therefore, the conversion factor (cf) was calculated according to the following equation:

$$cf\ (gO_2/g) = \frac{b \times M(O_2)}{a \times M(compound)} \qquad (9.4)$$

The conversion factors were 1.07 $gO_2\ g^{-1}$ for glucose, xylose, lactic and acetic acids, 1.51 $gO_2\ g^{-1}$ for propionic acid, 1.82 $gO_2\ g^{-1}$ for butyric acid, and 2.04 $gO_2\ g^{-1}$ for valeric acid. For biomass, it was assumed an empirical molecular formula of $C_5H_7NO_2$ that corresponded to a conversion factor of 1.42 $gO_2\ g^{-1}$ for biomass (Queirós et al., 2014).

The total acidification degree (AD_{Total}) represents the amount of substrate consumed to produce SCOA considering all the organic matter entering the reactor (Equation 9.5). The sugars acidification degree (AD_{Sugars}) represents the two main sugars consumed to produce SCOA (considering the xylose and glucose fed to the reactor) (Equation 9.6). These parameters were presented as percentages.

$$\text{AD}_{\text{Total}}(\text{gCOD/gCOD}) = \frac{[\text{SCOA}]}{\text{COD}_{\text{in}}} \times 100 \qquad (9.5)$$

$$\text{AD}_{\text{Sugars}}(\text{gCOD/gCOD}) = \frac{[\text{SCOA}]}{\text{COD}_{\text{sugars}}} \times 100 \qquad (9.6)$$

For the effluent, the yield on SCOA was calculated relatively to fed COD, represented by $Y_{SCOA/S}$ (Equation 7) and relatively to consumed sugars (xylose and glucose), $Y_{SCOA/Sugars}$ (Equation 8).

$$\text{Y}_{\text{SCOA/S}}(\text{gCOD/gCOD}) = \frac{[\text{SCOA}]_{\text{produced}}}{(\text{COD}_{\text{In}} - [\text{SCOA}]_{\text{in}}) - (\text{COD}_{\text{out}} - [\text{SCOA}]_{\text{out}})} \qquad (9.7)$$

$$\text{Y}_{\text{SCOA/Sugars}}(\text{gCOD/gCOD}) = \frac{[\text{SCOA}]_{\text{produced}}}{\text{COD}_{\text{Sugarsin}} - \text{COD}_{\text{Sugarsout}}} \qquad (9.8)$$

The substrate consumption volumetric rate ($-r_s$) and the SCOA production volumetric rate (r_p) in gCOD L^{-1} h^{-1} were also calculated by dividing the substrate consumed or product formed, respectively, by the HRT.

9.3. Results and Discussion

9.3.1. Continuous stirred tank reactor without pH control (CSTR-wo/pH)

The possibility of obtain SCOA from the acidification of HSSL was proved in chapter 8. Moreover, it was used an aerobic inoculum to select an acidogenic population. In this way, it was possible to bypass the possible growth of methanogenic microorganisms (Gerardi, 2003), which use the SCOA as substrate to produce methane and carbon dioxide and, consequently, affect negatively the yield of the process. The inocula used in the present work were activated sludge from an aerobic tank of a WWTP or MMC already adapted to anaerobic conditions. The inoculum used in the CSTR-wo/pH came from an acidogenic reactor, already enriched in acidogenic microorganisms, and had a concentration of 2.65 gVSS L^{-1}. CSTR-wo/pH started to work under similar conditions as the initial acidogenic reactor: HRT of 2.34 days, OLR of 7.62 gCOD L^{-1} d^{-1} and temperature of 30 °C (chapter 8). The substrate used as carbon source was the same, HSSL. The feed presented a COD concentration of 17.8 g L^{-1}, being 0.35 gCOD L^{-1} glucose, 2.58 gCOD L^{-1} xylose and 0.97 gCOD L^{-1} acetic acid.

The acidogenic fermentation of HSSL in the CSTR-wo/pH system lasted 262 days. The HRT1 (2.34 d) was applied until the 181st day, when the retention time was changed to 3 days (HRT2) during the last 81 days of reactor operation. The SCOA distribution, sugars and pH variations in the reactor are shown in Fig. 9.1. It is noteworthy that all graphical representation of SCOA in this study, display the SCOA produced in the process and not the total SCOA, which account the acetic already present in HSSL. As can be seen, the first 35 days were described by a major instability in the SCOA production and sugars consumption. Between days 17 and 21, an increase in pH was observed, which was caused by the addition of a new feed whose pH was not adjusted to 6.0 as it should. This variation had a considerable impact on the SCOA produced since acetic acid stopped being produced until the system pH was reestablished. The next days were followed by a

sharp increase in SCOA concentration.

A PSS was defined as a stage of the process where the system remained relatively stable, with small variations in the SCOA produced. A first PSS (PSS1) was considered to be achieved on day 41 for HRT1. The last days of the reactor operation at HRT1 were marked by major variations in the SCOA production and sugars consumption. Because of this instability, HRT was increased to 3 days, and the system took only 15 days to reach a more stable production and a second PSS (PSS2) was achieved on day 196.

The first days of fermentation, until PSS1, were characterized by a significant production of lactic acid. Between days 17 and 21, in which the pH increased, lactic acid was the only SCOA produced. That may indicate that the production of lactic acid could be associated with instability periods or perturbations of the system, like the start of the process or modifications on the fermentation parameters as already observed by Gouveia et al. (2016) and Temudo et al. (2007). Duque et al. (2014) also observed this pattern in lactic acid production during adaptation stages and temperature perturbations in the acidification of cheese whey and sugarcane molasses. During the PSS1, lactic acid was produced in a small extent, probably due to small drops in the pH, since it is known that lower pH values benefit its production (Itoh et al., 2012; Wu et al., 2015).

Results obtained for the CSTR-wo/pH showed long periods of instability at HRT of 2.34 days. During these periods, readily degradable sugars were not being totally consumed, and SCOA production obtained was rather lower when compared to the results obtained by in chapter 8. Then, HRT was increased to 3 days, lowering the OLR to 5.95 gCOD L^{-1} d^{-1}. In this way, it was possible to improve not only sugars consumption but also SCOA production as well as the stabilization of the system (Fig. 9.1). This decision was crucial considering that the CSTR-wo/pH was supplying fermented HSSL to a sequencing batch reactor to select a PHA-producing MMC, requiring a maximization of the amount of SCOA produced.

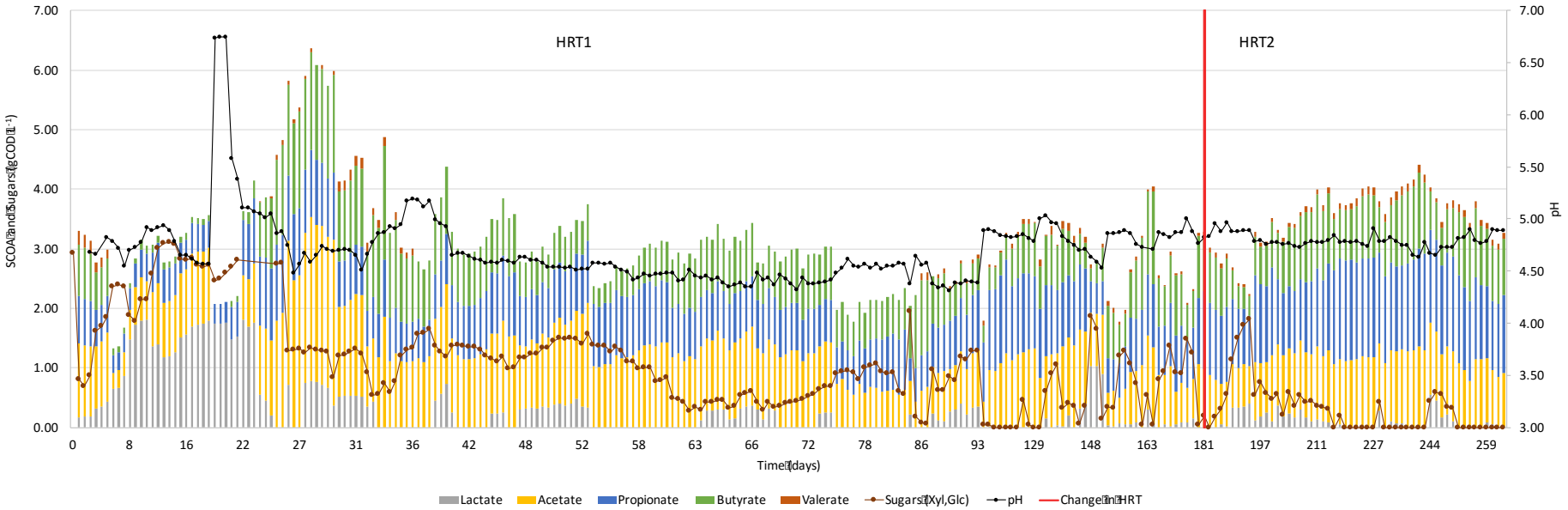

Fig. 9.1. Production of SCOA and its distribution during operations, along with the substrate (xylose and glucose) and pH variation over the fermentation time for CSTR-wo/pH.

During the operation at HRT1, the maximum concentration of SCOA achieved was 6.37 gCOD L^{-1} on day 28, corresponding to the maximum AD_{total} of 35.7% and AD_{sugars} of 217.0%. The operation at HRT2, on the other hand, presented a highest SCOA concentration of 4.40 gCOD L^{-1}, corresponding to the maximum AD_{total} of 24.7% and AD_{sugars} of 150.0% on day 239. Despite the lower maximum values, the operation at HRT2 was more stable since the average values were higher than during operation at HRT1. The average value of AD_{sugars} achieved for HRT2 was 121 ± 16.5% while for HRT1 was 107.1 ± 30.1% and the average SCOA produced was 3.10 ± 0.91 gCOD L^{-1} for HRT1 and 3.53 ± 0.48 gCOD L^{-1} for HRT2. In both periods, the acidification degrees relatively to sugars were higher than 100%, meaning that probably other components of the HSSL were being consumed along with xylose and glucose. Although, xylose and glucose were not the only sugars in HSSL, other sugars, namely ramnose, arabinose, mannose and galactose, accounted for 7.06 gCOD L^{-1} of pre-treated HSSL (data provided by Caima). However, due to the dilution of the HSSL in feeding, their concentration in the CSTR was lower than the detection limit of HPLC. Additionally, a significant part of dissolved carbohydrates, up to 25%, could be present in HSSL as oligosaccharides (Pereira et al., 2012; Xavier et al., 2010). During AF, the oligosaccharides could be hydrolyzed into its monomers and converted to SCOA. The average $Y_{SCOA/Sugars}$ achieved for HRT1 was 1.75 gCOD $gCOD^{-1}$ and for HRT2 1.35 gCOD $gCOD^{-1}$, these values were consistent with the AD_{sugars} obtained. Considering the total soluble COD, an average $Y_{SCOA/S}$ of 0.78 gCOD $gCOD^{-1}$ and 0.89 gCOD $gCOD^{-1}$ were obtained for HRT1 and HRT2, respectively (Table 9.1). Considering the average AD_{total}, 17.4 ± 4.8% and 19.8 ± 2.7% were achieved for HRT1 and HRT2, respectively, which were significantly below the maximum AD reported above, 35.7% and 24.7%. The results for the average AD_{total} achieved were low when compared to the literature. For instance, Arroja et al. (2012) achieved an AD ranging from 30 – 65% for four different substrates, sugarcane molasses, spent coffee grounds, dairy processing fatty slurry and cheese whey. The authors considered that the type of waste, along with the operational parameters imposed, such as HRT, OLR, pH and temperature, had a major effect on the success of the acidification process, and consequently, the AD achieved (Arroja et al., 2012). Silva et al. (2013) tested eight different substrates and achieved a

maximum AD of 51.6% for cheese whey, which corresponded to a maximum SCOA of 3.37 gCOD L^{-1}, followed by an AD of 42.1% and maximum SCOA of 3.11 gCOD L^{-1} for sugarcane molasses. Considering the maximum SCOA achieved, the value obtained in this work, 6.37 gCOD L^{-1}, and the average SCOA concentration achieved for HRT2, 3.53 ± 0.48 gCOD L^{-1}, were higher than the values obtained by Silva et al. (2013) for the eight effluents tested. Also, the average AD_{total} achieved for CSTR-wo/pH was only lower than three of the effluents tested, namely cheese whey, sugarcane molasses and organic fraction of municipal solid wastes. This is a significant result considering the composition of HSSL, rich in recalcitrant compounds, hardly biodegradable with the retention times applied, contrary to the effluents used by Silva et al. (2013). The effluents possessed mainly lactose and proteins, carbohydrates, and residues with relatively high biodegradability characteristics due to the presence of food wastes. Bengtsson et al. (2008) also tested four potential wastewaters to produce SCOA in batch experiments and observed a maximum AD of 67% for whey permeate and 66% for an effluent from a paper mill, respectively. Regarding SCOA concentration, Bengtsson et al. (2008) achieved a maximum value of 3.96 gCOD L^{-1} SCOA with paper mill wastewater at the end of the batch operation, comparable to those obtained in this work with HSSL in a continuous operation.

9.3.1.1. CSTR-wo/pH SCOA distribution

SCOA distribution was calculated considering only the SCOA produced. Considering the HRT1 and PSS1, the SCOA profile obtained was 4.43±6.56/36.7±6.00/32.1±6.75/26.0±7.79/0.86±1.32% for lactic, acetic, propionic, butyric and valeric acids, respectively. Regarding HRT2 and PSS2, the profile achieved was 2.90±3.51/29.9±1.74/38.3±4.28/26.2±4.62/2.73±0.51% (Table 9.1). During the operation at HRT1, although acetic acid was the main SCOA produced, propionic acid also showed a high percentage, followed by butyric acid. Considering the operation at HRT2, propionic acid was the main SCOA produced, followed by acetic and butyric acids, respectively. Also, at HRT2 the percentage of valeric acid present increased.

In general, the principal SCOA produced were acetic, propionic and butyric acids, which agreed with results reported in the literature (Bengtsson et al., 2008; Jiang et al., 2013; Jie et al., 2014; Yu and Fang, 2002). As mentioned before, lactic acid was produced during the instability stages, in higher concentrations during the start of the fermentation and decreasing in the following days. Also, valeric acid was produced almost in negligible concentrations with an average of 0.86 ± 1.32% and 2.73 ± 0.51% for HRT1 and HRT2, respectively. This was expected since valeric acid is not normally produced in this type of process or it is produced in minor amounts (Jankowska et al., 2015; Temudo et al., 2007; Yu and Fang, 2002). Some authors stated that while acetic, propionic and butyric acids can be produced directly from the fermentation of soluble proteins, carbohydrates and lipids, valeric acid is mainly produced from proteins degradation (Wang et al., 2014; Yu and Fang, 2002). Furthermore, Liang and Wan (2015) used brewer's spent grain demonstrated that under neutral conditions lactic acid and ethanol were consumed to produced SCOA. By adding lactic acid to the reactor feeding, Liang and Wan (2015) observed that lactic acid consumption was related mainly to the production of propionic and butyric concentrations, but also to valeric and caproic acids in smaller amounts. Therefore, these authors considered that although carbohydrates are the main substrate for SCOA production, lactic acid can also be used as electron donor to allow the production of longer SCOA (Liang and Wan, 2015). Although the pH values obtained in CSTH/pH were not neutral and the substrate was diverse, the production of valeric acid normally occurred simultaneously with lactic acid production or right after these periods. Moreover, Zoetemeyer et al. (1982) considered that pH values like those observed in CSTR-wo/pH might favor the production of longer chain acids since under extremely acidic conditions there were more reducing equivalents available to be assimilated into the acid chain.

Fig. 9.2 shows a clear shift between propionic and butyric acids concentration during the entire operation. A similar shift was already observed by Cohen et al. (1984) during AF of synthetic wastewater: a higher butyric acid production was achieved when a lower formation of propionic acid was found and vice versa with high acetic production in both situations. Authors also reported that a high production of butyric acid correlated

positively with the production of hydrogen and carbon dioxide, while a high production of propionic acid correlated negatively with little or none production of hydrogen (Cohen et al., 1984). The same tendency was verified by Bengtsson et al. (2008) for whey and paper mill wastewaters. A shift between the production of propionic and butyric acids was also observed and authors explained as a result of the competition between two bacterial communities: propionate and butyrate fermentation type bacteria (Bengtsson et al., 2008). The dominance of one of the bacterial groups could be related to reactor operation resulting in the production of propionic or butyric acids.

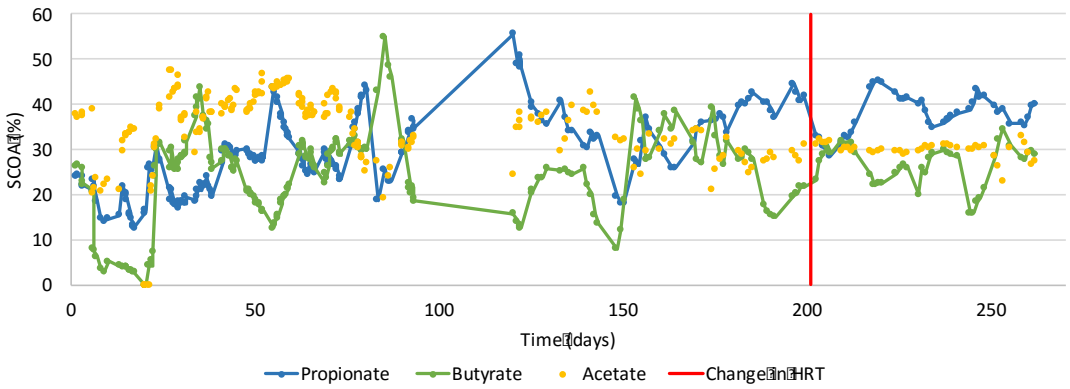

Fig. 9.2. Evolution of acetic, propionic and butyric acids concentration along CSTR-wo/pH operational time.

Table 9.1. Summary of the results from the CSTR-wo/pH for HRT1 and HRT2, CSTRpH for pH 6, 7 and 8 and MBBR.

Reactor	HRT	pH	Consumed Substrate	SCOA produced	Maximum SCOA produced	$Y_{SCOA/Sugars}$	$Y_{SCOA/S}$	r_s	r_p	AD_{sugars}	AD_{total}	Maximum AD_{total}	PSS	SCOA PSS profile
CSTR-wo/pH	2.34	-	1.77 ± 0.79	3.10 ± 0.91	6.37	1.75	0.78	0.025 ± 0.011	0.043 ± 0.012	107.1 ± 30.1	17.4 ± 4.8	35.7	41	4.43±6.56/36.7±6.00/32.1±6.75/26.0±7.79/0.86± 1.32
	3	-	2.62 ± 0.43	3.53 ± 0.48	4.40	1.35	0.89	0.030 ± 0.006	0.049 ± 0.007	121 ± 16.5	19.8 ± 2.7	24.7	15	2.90±3.51/29.9±1.74/38.3±4.28/26.2±4.62/2.73±0.51
CSTRpH	3	6	2.90 ± 0.03	2.36 ± 0.50	2.98	0.82	0.73	0.040 ± 0.0003	0.033 ± 0.007	80.8 ± 17.3	13.2 ± 2.82	16.7	17	0.01±0.06/57.8±7.71/27.0±5.84/14.8±5.23/0.36±0.53
	3	7	2.93	2.38 ± 0.11	2.67	0.81	0.73	0.040	0.033 ± 0.002	81.6 ± 3.81	13.3 ± 0.62	15.0	25	1.32±1.61/82.7±1.97/1.28±0.40/14.9±2.43/0.11±0.20
	3	8	2.93	2.27 ± 0.12	2.47	0.78	0.73	0.040	0.031 ± 0.002	78.0 ± 4.24	12.8 ± 0.71	13.9	17	1.55±1.69/89.2±2.54/1.88±0.96/7.20±2.21/0.26±0.49
MBBR	3	-	2.71	2.32 ± 0.56	2.85	0.86	0.73	0.037 ± 0.004	0.032 ± 0.006	79.5 ± 13.8	13.0 ± 2.26	16.0	26	0.00/23.0±5.70/15.9±4.75/59.9±7.93/1.21±0.21

Units: HRT – days; Consumed substrate – gCOD L^{-1}; SCOA produced – gCOD L^{-1}; $Y_{SCOA/Sugars}$ – gCOD $gCOD^{-1}$; $Y_{SCOA/S}$ – gCOD $gCOD^{-1}$; $-r_S$ – gCOD L^{-1} h^{-1}; r_P – gCOD L^{-1} h^{-1}; AD_{Sugars} – %; AD_{Total} – %; Maximum AD_{Total} – %; PSS – days needed to achieve PSS; SCOA PSS profile – % (Lactic/Acetic/Propionic/Butyric/Valeric acids).

9.3.2. Continuous stirred tank reactor with pH control (CSTRpH)

The study was done to evaluate the effect of pH on SCOA distribution and SCOA concentrations obtained in a CSTR system controlled at three different pH values: 6, 7 and 8. The choice of the pH values was based on the results of preliminary batch tests of HSSL (data not shown). These tests revealed more interesting SCOA profiles at this pH values. The conditions imposed to this reactor were the same as those imposed to CSTR-wo/pH, choosing a HRT of 3 days and with pH control. An OLR of 5.95 gCOD L^{-1} d^{-1} was imposed, at a temperature of 30 °C. The inoculum used was fresh sludge that came from an aerobic tank of WWTP, with a concentration of 11.6 g L^{-1}, since biomass withdrawn from CSTR-wo/pH performed poorly during preliminary batch tests (data not shown).

The CSTRpH operation was carried out at pH 6 during 77 days, pH 7 during 35 days, and pH 8 during 31 days, resulting in a global operational period of 143 days. SCOA distribution and SCOA and substrate concentrations CSTRpH are shown in Fig. 9.3. In contrast with the 41 days of instability before reaching PSS reported in the CSTR-wo/pH, CSTRpH at pH 6 took only 17 days to reach a PSS with a gradual increase on SCOA production. Moreover, the PSS achieved for CSTRpH was more stable than the one for CSTR-wo/pH, since lower variation on the concentration and SCOA profiles were observed. In previous works, Horiuchi et al. (2002) and Jankowska et al. (2015) verified that the pH control had a strong influence in the process stability (Horiuchi et al., 2002; Jankowska et al., 2015). SCOA concentrations remained quite stable even when the pH of CSTRpH changed from 6 to 7 and 7 to 8.

Xylose and glucose were almost fully consumed at pH 6, with residual concentrations remaining in the CSTR during some periods. At pH 7 and 8 the two sugars were completely consumed. Noteworthy, the residual production of lactic acid that could be due a short period of instability and higher pH tested.

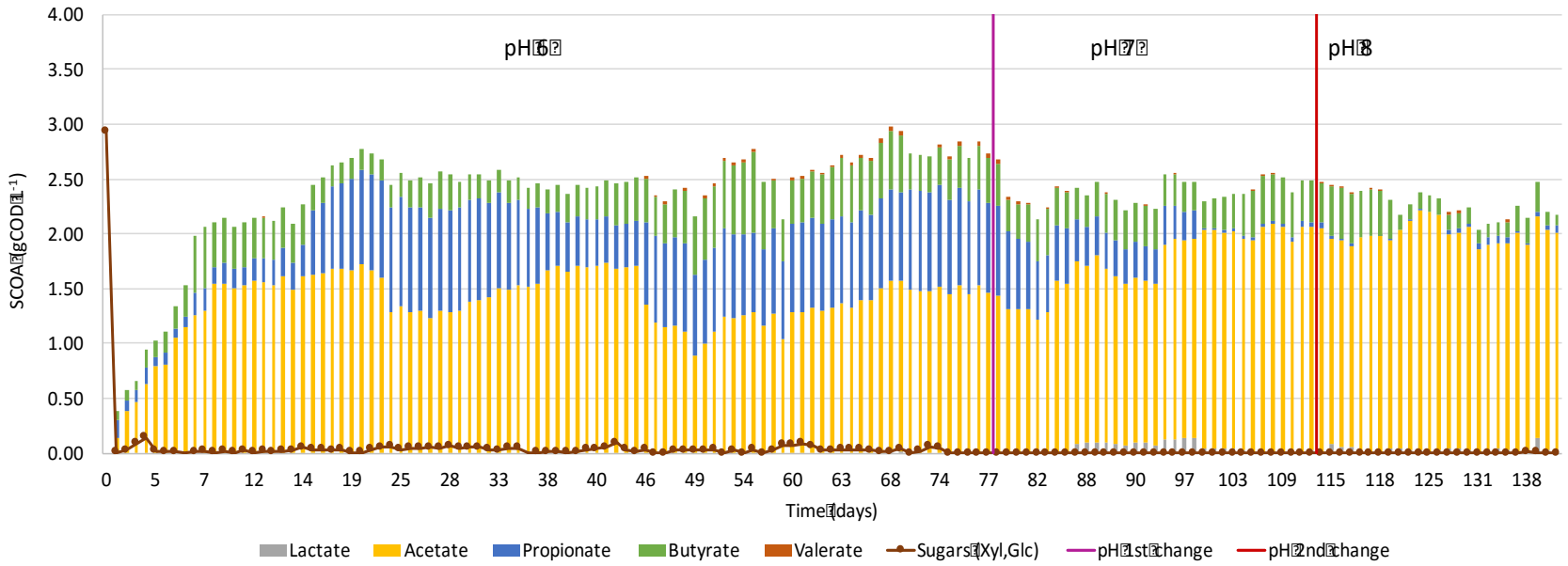

Fig 9.3. Production of SCOA and its distribution during operations, along with the substrate (xylose and glucose) over the fermentation time for CSTRpH for the three pH values tested.

The maximum SCOA achieved for pH 6, 7 and 8 were 2.98, 2.67 and 2.47 gCOD L^{-1}, respectively. These values corresponded to the maximum AD_{total} of 16.7%, 15.0% and 13.9% (Table 9.1). All the values achieved were quite lower than the maximum values of achieved for the CSTR-wo/pH, 4.40 gCOD L^{-1} that corresponded to a maximum AD_{total} of 24.7%, for the same HRT. Considering the average values, the maximum SCOA values obtained for CSTRpH were also lower than the value achieved for the system without pH control at an HRT of 3 days, as can be seen in Table 9.1. Comparing yields, $Y_{SCOA/Sugars}$ achieved were higher for pH 6 and 7 with 0.82 gCOD $gCOD^{-1}$ and 0.81 gCOD $gCOD^{-1}$ while 0.78 gCOD $gCOD^{-1}$ were achieved for pH 8. Considering the whole soluble COD available, the $Y_{SCOA/S}$ was the same for the three pH values, 0.73 gCOD $gCOD^{-1}$.

Comparing CSTR-wo/pH at HRT2 and CSTRpH, SCOA concentrations obtained for pH 8 in last system were the lowest. This, consequently, resulted in a low average AD_{total}, 13.2 ± 2.8%, when compared to the 19.8 ± 2.7% obtained for CSTR-wo/pH. The low SCOA concentration obtained could be explained by the deviation of carbon to the production, in substantial amounts, of other metabolites that were not yet identified. From HPLC chromatogram analysis aroused the hypothesis of this compound to be succinic acid. Also, the production of this acid was already reported by Jankowska et al. (2015), from primary and waste activated sludge in which succinate was the dominant acid at pH 6, and by Temudo et al. (2007) from glucose at a pH higher than 6. Lim et al. (2008) reported the production of small amounts of succinate at pH 5.5 and higher quantities at pH 5. Succinic acid was the main product (44 – 48%) of food waste fermentation (Lim et al., 2008). In the current work, the production of the unknown metabolite was only verified for pH 6 and neither for pH 7 nor 8. Thus, the identification of the unknown compound should be accomplished in the future. The possibility of succinate production via AF it is an important feature since it is one of the most important chemical compounds identified by the U.S. Department of Energy's to be produced from renewable biomass (Werpy and Petersen, 2004). Moreover, further work should include CSTR operation at pH 5, 5.5, 6.5 and 7.5 to verify the difference between the profiles of SCOA obtained and respective concentration.

9.3.2.1. CSTRpH SCOA distribution

The different pH values applied during reactor operation did not seem to affect the overall concentration of SCOA but clearly interfered with their distribution. While SCOA distribution remained relatively stable during the operational period at pH 6, at the pH 7, lactic acid was produced in small quantities along with the decrease of propionic acid. Butyric acid production remained relatively stable between pH 6 and 7, 14.8 ± 5.23% and 14.9 ± 2.43% of total SCOA. However, butyric acid decreased during the operation at pH 8 to 7.20 ± 2.21%. Temudo et al. (2007) also observed that pH values lower than 6 favored butyric acid production, from glucose fermentation. Also, Jankowska et al. (2015) reported the decrease in butyric acid production with the increase of pH, due to the dominance of facultative anaerobic bacteria which lack enzymes involved in butyric acid production. Lim et al. (2008) observed the increase of acetic acid concentration with pH increase, but testing only three pH values, being them 5, 5.5 and 6. They achieved a higher acetic acid production at pH 6 (Table 9.2). As can be seen in Fig. 9.3 and Table 9.1, pH 6 favored the propionic acid production, with 27.0%, when compared to pH 7 and pH 8, with 1.28% and 1.88%, respectively. However, these results were lower than of obtained in CSTR-wo/pH at HRT2. The average propionic acid was 38.3% and the pH stayed under 5.0 the whole operational period. This disagrees with the findings of Horiuchi et al. (2002) that submitted a stable culture during glucose fermentation to pH variations and observed that some propionic bacteria have an optimal pH of 7 - 8 for cell growth.

Table 9.2 summarizes several results from the literature that studied the influence of pH within the range 4 – 7 in AF. There is no clear tendency between the SCOA profiles and the imposed pH. Such comparison should be only done between studies with the same substrate because, apparently, SCOA profiles produced seem to be more dependent on substrate composition than on pH (Lee et al., 2014).

Table 9.2. Review of some results reported regarding AF of various wastes. SCOA profiles are presented in the order lactic/acetic/propionic/butyric/valeric acids.

Substrate	Composition	System	HRT	OLR	T (°C)	pH	SCOA	SCOA Profiles (%)	Study
Sugar cane molasses	Mainly sucrose and fructose	CSTR 1.14 L	10 h	35 Cmmol L^{-1} h^{-1}	30	5	238 Cmmol L^{-1}	3/36/14/28/22	Albuquerque et al. (2007)
				31 Cmmol L^{-1} h^{-1}		6	194 Cmmol L^{-1}	0/63/17/14/7	
				37 Cmmol L^{-1} h^{-1}		7	209 Cmmol L^{-1}	7/50/26/11/5	
Food waste (simulated)	Rice 35%, cabbage 45%, pork 16%, tofu 4%	Batch 4.5 L	8 d	-	35	Uncontrolled	3.94 g L^{-1}	0/67.0/3.7/29.4/0	Jiang et al. (2013)
						5	17.1 g L^{-1}	0/60.4/8.3/31.1/0.15	
						6	39.5 g L^{-1}	0/23.8/13.5/53.3/9.5	
						7	37.1 g L^{-1}	0/34.1/19.7/42.7/3.6	
Food waste (synthetic)	Glucose	Semi-continuous 2 L	8 d	9 g $L^{-1}d^{-1}$	35	5	18.0 g L^{-1}	0/18.2/4.4/18.4/0	Lim et al. (2008)
						5.5	24.0 g L^{-1}	0/33/28.1/21.6/14.3	
						6	25.5 g L^{-1}	0/50.9/25.1/21.5/7.0	
Cheese whey	Lactose 78.4%, proteins 13.6%, fats 1.2%	CSTR + membrane filtration module 1.14 L	1 d	13.7 gCOD $L^{-1}d^{-1}$	37	6	9.7 gCOD L^{-1}	4/61/13/12/1	Duque et al. (2014)

Sugar cane molasses	Sugars 54%			16.3 gCOD $L^{-1}d^{-1}$			13.2 gCOD L^{-1}	0/24/34/16/24	
Cheese whey	Lactose 78.4%, proteins 13.6%, fats 1.2%	CSTR 1.25 L	1 d	15.9 gCOD L^{-1} d^{-1}	30	4.5	11.0 gCOD L^{-1}	80/18/0/1/0	Gouveia et al. (2016)
						5	14.1 gCOD L^{-1}	67/30/2/0/0	
						6	13.7 gCOD L^{-1}	48/40/10/2/0	
						7	12.7 gCOD L^{-1}	56/37/6/0/0	
HSSL	LS 70.7%, xylose 14.4%, glucose 2%	CSTR 1.55 L	1.76 d	11.8 gCOD L^{-1} d^{-1}	30	Uncontrolled	5.50 gCOD L^{-1}	5.7/53.6/22.0/18.7/0	Chapter 8
	LS 70.7%, xylose 14.4%, glucose 2%	CSTR 2 L	2.34	7.62 gCOD L^{-1} d^{-1}	30	Uncontrolled	3.10 gCOD L^{-1}	4.43/36.7/32.1/26.0/0.86	Present study
			3 d	5.95 gCOD L^{-1} d^{-1}			3.53 gCOD L^{-1}	2.90/29.9/38.3/26.2/2.73	
			3	5.95 gCOD L^{-1} d^{-1}		6	2.36 gCOD L^{-1}	0.01/57.8/27.0/14.8/0.36	
						7	2.38 gCOD L^{-1}	1.32/82.7/1.28/14.9/0.11	
						8	2.27 gCOD L^{-1}	1.55/89.2/1.88/7.20/0.26	
		MBBR 3.22 L				Uncontrolled	2.32 gCOD L^{-1}	0.00/23.0/15.9/59.9/1.21	

9.3.3. Moving Bed Biofilm Reactor

MBBR system allows combining suspended growth with fixed biofilm processes, making use of the best characteristics and advantages of a CSTR system, without being restrained by its disadvantages (Chai et al., 2014; Oliveira et al., 2014). These systems have been used in the past few years in the treatment of effluents (Borkar et al., 2013; Chai et al., 2014; Oliveira et al., 2014), but the study of AF using MBBR is limited. Imposing low pH values to AF reactors does not require reagents and further equipment, thus lowering the process costs, which is crucial for scale-up processes. However, reactors that operate at low pH require a moderately long SRT due to inhibition of microbial growth and the need to deviate energy to the maintenance of the intracellular pH, which could be difficult to obtain in a CSTR (Tamis et al., 2015). Moreover, low pH values have a negative effect on the conversion of sugars into SCOA as demonstrated by CSTR-wo/pH results. The use of a reactor with a biomass retention system, such MBBR, could be a strategy to avoid these problems.

The MBBR used in this work was operated without pH control and, the operational parameters imposed were the same as those imposed to CSTR-wo/pH during the period at HRT2. The carriers chosen to perform the acidification of HSSL in the MBBR system were bioflow 9, due to its characteristics, namely diameter, low density and high specific surface area. Moreover, Chai et al. (2014) compared the performance of bioflow 9 and bioflow 30 on the treatment of winery wastewaters and showed that the former was more efficient in the attachment of biomass. The percentage of carriers used, 41%, was chosen taking into account the work of Arroja et al. (2012) and despite some authors used filling fractions between 60 and 70% of carriers for the treatment of wastewaters (Chai et al., 2014; Sheli and Moletta, 2007; Wang et al., 2009). The decision was based on the high density of the medium, which could result in difficulties in stirring the medium if a high percentage of carriers was used.

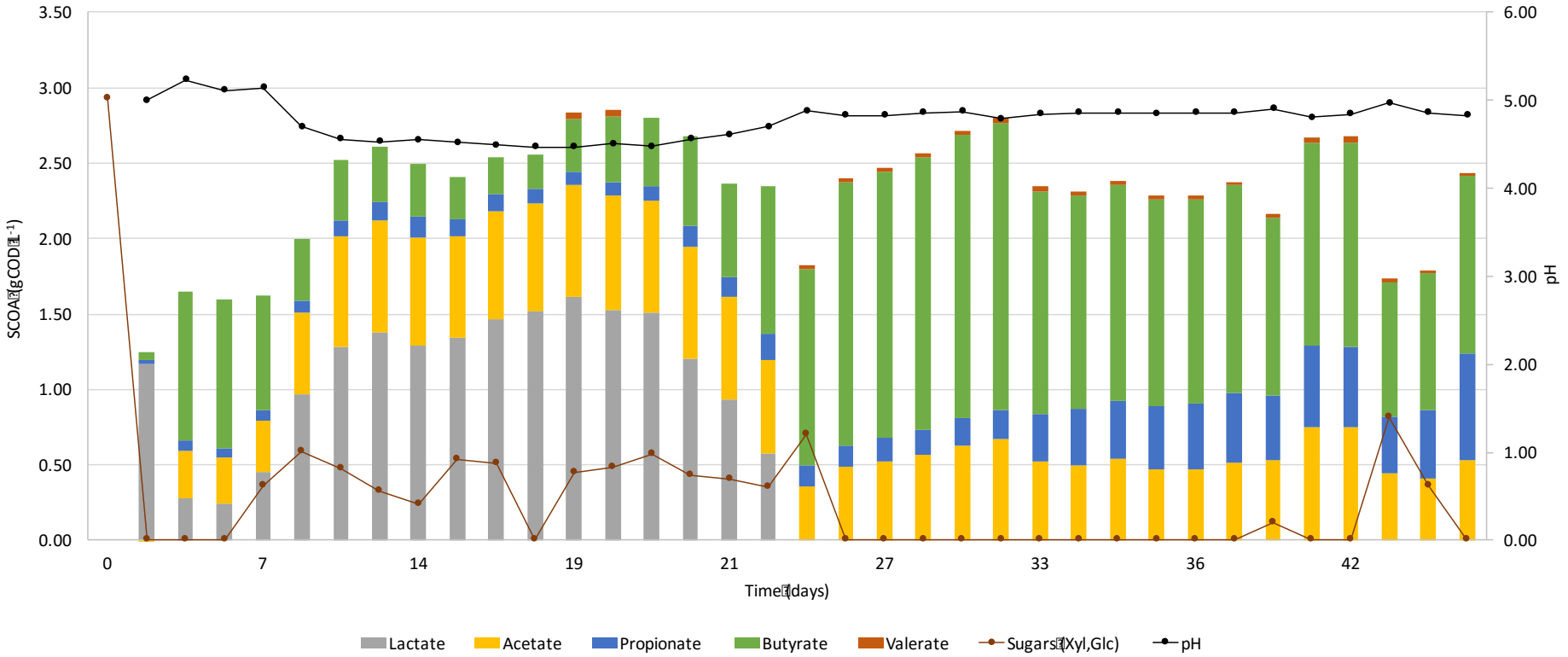

Fig. 9.4. Production of SCOA and its distribution during operations, along with the substrate (xylose and glucose) over the fermentation time for MBBR system.

The MBBR was operated for the AF of HSSL for 47 days. SCOA distribution, substrate concentration and pH variations in the SBR are shown in Fig. 9.4. The first 26 days were marked by system instability with variations in SCOA concentrations along with significant production of lactic acid. As already seen in CSTR-wo/pH system, lactic acid could be a result of an unstable AF system. During this period, consumption of the sugars was not complete. Nevertheless, it took less time, 26 days, to reach a stable performance than CSTR-wo/pH, regarding SCOA concentration and profile (Table 9.1). After this period SCOA production remained stable (Fig. 9.4), confirming that MBBR system is known to remain stable and with good performances when high OLR values are applied (Karadag et al., 2015).

The maximum SCOA produced in MBBR was 2.85 gCOD L^{-1}, less than half of the value achieved in CSTR-wo/pH, but higher than the values obtained for CSTRpH for pH 7 and 8 (Table 9.1). Considering the yields, a $Y_{SCOA/Sugars}$ of 0.86 gCOD $gCOD^{-1}$ achieved for in MBBR was lower than those achieved for CSTR-wo/pH, 1.75 and 1.35 gCOD $gCOD^{-1}$ but higher than the values obtained for CSTRpH for the three pH values tested. Considering $Y_{SCOA/S}$ a value of 0.73 gCOD $gCOD^{-1}$ was determined, the same as those achieved for CSTRpH for all the pH values, and lower than the 0.78 and 0.89 gCOD $gCOD^{-1}$ achieved for CSTR-wo/pH. The average AD_{Sugars} achieved in MBBR was 79.5 ± 13.8%, lower than the values for CSTR-wo/pH and CSTRpH for pH 6 and 7. The AD_{total} was 13.0 ± 2.26% with a maximum value of 16.0% obtained on day 20 and corresponded to the maximum SCOA concentration achieved.

Regarding SCOA distribution, the MBBR configuration showed a considerable impact on the profile obtained. This influence was verified specially in propionic and butyric concentrations, showing once more the shift between these two SCOA, as observed in the previous systems. Noteworthy, this was the first time that acetic acid did not correspond to the acid produced in higher amount in this study, being the MMBR system dominated by butyric acid production. The higher butyric acid concentrations could be associated with two possible factors: although slightly higher, pH was kept stable between 4.8 and 4.9, while in CSTR-wo/pH it varied from 4.3 to 4.6. Also, considering that

different bacterial groups could produce either butyric or propionic acid, the butyrate-producing bacteria could attach quicker to the carriers dominating the MBBR population.

Considering the experimental results and the literature (Escudié et al., 2011), testing higher OLR appears to be a crucial parameter to enhance the potentialities of this MBBR system for AF. One advantage of biofilm reactors is the capacity to tolerate high OLR, thus enhancing the productivity of the system (Escudié et al., 2011; Karadag et al., 2015), since more influent is converted for unit of time. Also, since microorganisms are attached to a support, they could resist easily to organic load shocks, SCOA accumulation in medium or even to inhibitors of the influent, especially when acidifying more recalcitrant wastes as HSSL. Moreover, it is also important to test other operational parameters such as temperature and a pH control system. The comparison between a CSTR and MBBR systems with pH control at different values is crucial and must be done in near in the future.

9.4. Conclusion

In this work, several operational parameters were tested on AF of HSSL. The influence of HRT, pH and reactor configuration was assessed regarding the SCOA concentration, profiles and production stability.

In general, for all the experiments carried out, the main SCOA produced were lactic, acetic, propionic and butyric acids and, in minor amounts, valeric acid. The production of lactic acid was associated not only with the lower pH values verified in the systems but also with instability and adaptation phases of the AF reactors. Sugars consumption showed to have a relation with the pH, with the higher values favoring the complete fermentation. Furthermore, glucose was consumed preferentially to xylose, a fact associated with the metabolism of conversion of the two sugars. A shift between propionic and butyric acids was observed in all the systems tested and was associated with the dominance of propionic- or butyric-producing bacteria. Additionally, pH showed a major importance in the stability of the process and in the SCOA profiles achieved.

The MBBR conformation without pH control revealed a shorter start-up time when compared to CSTR-wo/pH. Despite the total amount of SCOA produced being lower

than CSTR-wo/pH, the production was by far more stable and dominated by butyric acid production. In this way, the reactor conformation revealed to have an impact on the profiles SCOA produced.

For all the experiments performed, AF was proven to be an efficient process that resulted in value-added chemicals as SCOA, allowing the valorization of an organic-rich stream, the HSSL.

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Chapter 10

Exploitation of different pulp and paper factory waste streams for PHA-production

Pulp and paper factories produce more than one residue that can be explored and valorize through PHA production. A rich and diverse SCOA-residue can be used to acclimatize an MMC. The culture will be able to consume the different acids present, and later, accumulate PHA with different composition from the different residues streams.

In this work, two streams are explored for PHA production: an SCOA enriched stream that resulted from the acidogenic fermentation of HSSL, and the so-called "condensate", rich in acetic acid with minor factions of furfural and methanol.

An MMC was acclimatized with acidified HSSL. The culture could adapt to several imposed conditions, revealing its robustness every time the operational parameters were changed. F/F was kept below or equal to 0.2, with a constant production of a copolymer of P(3HB-*co*-3HV). During the accumulation tests, the selected MMC responded well to the different streams used to maximize the PHA content and manipulate its composition. Two different copolymers of P(3HB-*co*-3HV), from acidified HSSL, and a homopolymer of P(3HB), from condensate, were obtained. From the stream used during the selection stage, the MMC could accumulate up to 74.4% cdw, with a volumetric productivity of 0.27 gPHA L^{-1} h^{-1}.

10.1. Introduction

A critical affliction of today's society is the amount of wastes produced every day and the resources applied to treat them. Nevertheless, not all wastes are duly processed, and many of them end up being discharged into the environment. Plastics are among those that persist for thousands of years and with a huge impact in all ecosystems. Alternatives to conventional plastics, mainly those completely biodegradable, have been receiving attention over the last 20 years. Among them, PHA stand out due to their wide range of thermoplastics properties(Laycock et al., 2013).

Unfortunately, the biggest obstacle to the commercialization of PHA is their high production cost. Many endeavors were done to lower PHA price: from new microbial strains and feeds (Guzik et al., 2014; Mendonça et al., 2014), to the use of metabolic engineering to upgrade bacterial strains and to the development of more efficient fermentations processes (Insomphun et al., 2014; Kawashima et al., 2015; Yin et al., 2015). Still, the price could be further trim if a treatment-valorization strategy of wastes and residues (surplus-based feedstocks) by MMC is adopted.

In general, the key for a successful PHA production by MMC is their enrichment in PHA-storing bacteria (Queirós et al., 2015; Serafim et al., 2008a). Usually, enrichment strategies take place under alternating anaerobic-aerobic or feast-famine conditions together with other operational parameters such as sludge and hydraulic retention time, pH, organic loading rate, C/N ratio, etc. The main struggle now is to increase the volumetric productivity since specific productivities, with polymer yields on substrate and maximum PHA contents similar to those attained by pure cultures were already shown possible to obtain (Johnson et al., 2009; Serafim et al., 2004).

Besides increasing volumetric productivities, it is also important to determine the quality and the reproducibility of the biopolymers produced by MMC. Such factors should meet the standards required for standard plastic applications particularly considering polymers produced from waste feedstocks. However, only a few studies focused their efforts on characterizing the polymers obtained by this kind of systems (Albuquerque et al., 2011; Morgan-Sagastume et al., 2010; Serafim et al., 2008b), and were even lower those presenting a detailed analysis (Laycock et al., 2013).

PHA monomeric composition, which determines the physical and mechanical properties of the final polymer, depends on the percentage of SCOA in the fermented feedstock produced in the early acidogenic fermentation and supplied to the MMC (Fig. 10.1). PHA production by MMC can be manipulated to produce copolymers with different composition and improved properties by controlling the operational conditions of the acidogenic reactor (Albuquerque et al., 2007; Lemos et al., 2006; Serafim et al., 2008b; Wang et al., 2013). Using diverse SCOA profiles, a wide range of PHA compositions can be obtained. MMC fed with fermented feedstocks, containing mixtures of acetic, propionic, butyric and valeric acids, can produce PHA with a considerably high diversity of different HA monomers, containing monomers other than 3HB, such as 3-hydroxyvalerate (3HV), 3-hydroxy-2-methyl-valerate (3H2MV) or 3-hydroxyhexanoate (3HHx) (Lemos et al., 2006; Pisco et al., 2009). Even so, the most common copolymer produced by MMC is P(3HB-*co*-3HV) in ADF systems, and therefore it is imperative to manipulate the type of carbon fed to the culture once different 3HV monomer proportion will be obtained and will define physical and chemical characteristics of P(3HB-*co*-3HV).

SCOA composition of feeding not only influences PHA composition but also the microbial population selected. Studies using synthetic acids revealed different behaviors by MMC when acclimatized to one of them and in batch tests fed with a different acid (Lemos et al., 2006; Wang et al., 2013; Chang et al., 2012). Lemos et al. (2006) observed that two distinct populations were selected when using acetic acid or propionic acid as sole carbon source, and behaved differently when the feed was shift. For the population adapted to propionic acid, a copolymer of P(3HB-*co*-3HV) was always obtained with acetic acid, propionic acid or a mixture of both substrates and for population selected with acetic acid, a homopolymer of P(3HB) was obtained from acetic and butyric acids and a terpolymer, P(HB/HV/HMV), was formed from propionic, valeric and a mixture of acetic and propionic acids (Lemos et al., 2006). Wang et al. (2013), confirmed such results, observing that propionate-acclimated sludge exhibited better PHA production from propionic acid than from acetic acid regarding kinetics and stoichiometry and acetate-acclimated sludge demonstrated a similar behavior when using acetic acid. These authors

also observed that mixed-carbon-acclimated sludge performed better in PHA production, including regarding rapid kinetics.

In this study, the possibility to control polymer composition and properties was assessed, by enriching an MMC in PHA-storing microorganisms able to consume a wide variety of SCOA. This study also intended to verify the possibility of using more than one by-product stream from a pulp and paper factory to produce different PHA, while a single selection reactor was operated (Fig. 10.1). Later, batch tests were done, under different nutrient-limiting condition and SCOA profiles to maximize the PHA content and manipulate the PHA composition. Both acclimatization and batches tests were done with HSSL fermented in the previous chapter through AF.

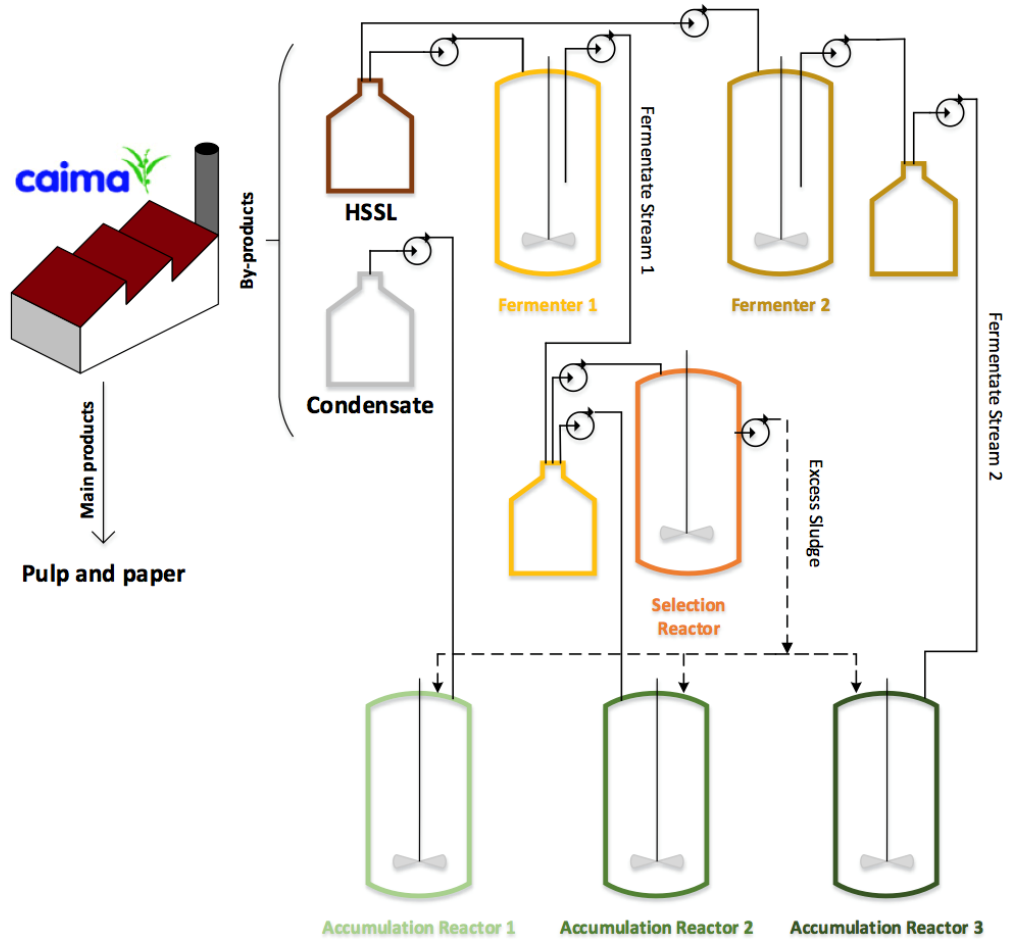


Fig. 10.1. Schematic of the strategy adopted to integrate the waste streams from CAIMA into PHA production.

10.2. Materials and Methods

10.2.1. Culture

The MMC used in this work was collected from an aerobic tank from the wastewater treatment plant SIMRia – Aveiro Sul.

10.2.2. Culture Media

Two of the main byproducts of Caima – Indústria de Celulose S.A. (Constância, Portugal), HSSL from magnesium based acidic sulfite pulping of *Eucalyptus globulus* and condensate stream, originated by successive evaporations of the HSSL, were used as substrate.

HSSL was used after submitted to an acidification step. Different conditions were used in the acidification process originating two distinct streams of acidified HSSL, A and B (chapter 9).

10.2.2.1. Stream A

Stream A was produced in an acidogenic CSTR system without pH control (chapter 9). The average SCOA profile of Stream A was 4.43/36.7/32.1/26.0/0.86% of lactic, acetic, propionic, butyric and valeric acids, respectively. The acidified effluent was submitted to biomass removal, pH adjusted to 6.5 and diluted with a mineral solution to obtain the desired concentration of SCOA for each period of operation of the SBR. The mineral solution composition was 0.160 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.080 g L^{-1} of $CaSO_4 \cdot 2H_2O$, 0.008 g L^{-1} of $Na_2MoO_4 \cdot 2H_2O$, 0.160 g L^{-1} of NH_4Cl, 0.4 g L^{-1} of CH_4N_2S. The medium was autoclaved for 20 min at 121 °C and afterward 0,016 g L^{-1} of KH_2PO_4 and 0,064 g L^{-1} of K_2HPO_4 were added under sterile conditions. This stream was used in both selection and accumulation steps.

10.2.2.2. Stream B

Stream B was produced in a system with controlled pH at 6 (chapter 9). The average SCOA profile achieved was 0.01/57.8/27.0/14.8/0.36% of lactic, acetic, propionic,

butyric and valeric acids, respectively. It was then submitted to the same procedures as Stream A. This stream was only used in the accumulation step.

10.2.2.3. Condensate

Condensate is mainly composed of acetic acid (16.5 g L^{-1}), methanol (0.84 g L^{-1}) and furfural (0.83 g L^{-1}). Before use, its pH was adjusted to 6.5, and it was diluted to obtain the desired concentration of acetic acid with a mineral solution. The mineral solution composition was 0.160 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.080 g L^{-1} of $CaSO_4 \cdot 2H_2O$, 0.008 g L^{-1} of $Na_2MoO_4 \cdot 2H_2O$, 0.160 g L^{-1} of NH_4Cl, 0.4 g L^{-1} of CH_4N_2S, 0,016 g L^{-1} of KH_2PO_4 and 0,064 g L^{-1} of K_2HPO_4. Condensate was only used in the accumulation step.

10.2.2.4. Matrix

The matrix was obtained by filtering the withdrawn medium from the selection reactor and supplemented with synthetic SCOA, namely acetic and propionic acids, to be used in the accumulation step.

10.2.3. Selection Bioreactor

The operation of the reactor lasted 156 days, to select a PHA-storing culture. The SBR working volume was 1.5 L and it was operated under ADF strategy, during which alternating feast and famine phase were imposed per cycle. For the first 25 days, the cycle length was 24h, comprising 22.5 hours of aerobiosis, 1 hour of settling (with agitation and aeration switched off), 0.5 hours of withdrawing (half of the reactor volume was removed) and, finally, the volume replacement (with fresh medium for period of 15 minutes). The HRT during this period was 2 days. Hereafter, the cycle length was reduced to 12 h, with 10.5 hours of aerobiosis. The HRT in this period was 1 day. Everything else was kept the same. During all the operation time, the SRT was kept at 5 days by purging 300 mL per day. Reactor stirring (400 rpm), aeration and feeding and withdrawing pumps were controlled with timers. Dissolved oxygen, temperature and pH were continuously monitored.

To prevent foam formation, diluted silicone anti-foam (1:20) was manually added when excessive foam was observed. The SBR was cleaned daily to prevent excessive biofilm formation on reactor walls and electrode surfaces.

The selection reactor had three distinct periods of operation as described in Table 10.1.

Table 10.1. Conditions imposed to the selection SBR

Period Designation	Operation Days	Cycle Length	OLR	SCOA	Feeding	HRT
P1	0 – 25	24	2.2	17.5	Stream A	2
P2	26 – 90	12	4.5	35	Stream A	1
P3	91 – 156	12	7.0	50	Stream A	1

Units: cycle length – h; OLR –gCOD L^{-1} d^{-1}; SCOA – Cmmol L^{-1}; HRT – days.

10.2.4. Batch Accumulations Tests

Several accumulation tests were conducted in a reactor BIOSTAT® A Plus-Sartorius, with a working volume of 4 L, without temperature control. A respirometer was coupled to the bioreactor and constantly stirred at 400 rpm. The medium was circulated by a peristaltic pump and DO registered. The reactor was stirred at 400 rpm and the values of pH, DO and temperature were monitored.

The inoculum used was collected from the SBR and aerated for 15 h to ensure the consumption of accumulated PHA along with residual ammonium. Tests were performed by feeding the system in a pulse-wise manner every time an abrupt increase in the DO was observed.

Several tests were conducted under different conditions (Table 10.2). At the end of each test, biomass was collected for PHA extraction.

10.2.5. Sampling

Concerning the selection SBR, samples were collected to monitor the overall performance of the cycles. pH, temperature and percentage of DO were registered. During accumulation tests, samples were collected every 10 minutes for VSS quantification and other analysis. Samples were centrifuged at 13000 rpm for 5 min. The

solid was separated from the supernatant and both frozen at -16 °C. The former was used for PHA determination and the later for determination of SCOA, LS, ammonium and COD.

Table10.2. Accumulations batches performed.

Assay	Substrate	$Cmmol_{SCOA}$/ pulse	Nº of Pulses	Time (h)	Limitation
AT1	Stream A	35	5	2.5	None
AT2	Stream A	35	5	8	Ammonium
AT3	Condensate	35	4	4.5	None
AT4	Condensate	35	5	5	Ammonium
AT5	Matrix + Acetic acid	35	5	4	None
AT6	Matrix + Propionic acid	35	4	8	None
AT7	Stream A	50	9	12	Ammonium
AT8	Stream B	50	8	12	Ammonium
AT9	Condensate	50	9	9.5	Ammonium

10.2.6. Analytical Methods

The ammonium concentration was followed using a Thermo Scientific Ion Selective Electrode. To 1 mL of the samples was added 20 µL of Ionic Strength Adjuster (ISA). This solution is composed of 5 M NaOH, 0.05 M EDTA, 10% methanol and provides a constant background ionic strength and adjusts the solution pH. The calibration was done resorting to a standard curve.

HPLC was used to determine the concentration of SCOA in the samples collected. The samples were filtered with 0.2 µm pore size filters. Then, 20 µL of sample was injected (Auto-sampler Hitachi L-2200) in an anion exchange column (Rezex™ ROA-Organic Acid H+ (8%)) connected to a refraction index detector (Hitachi RI L-2490). The column was at 65 °C in a Gecko 2000 external oven and the eluent used was 0.05 N H_2SO_4, prepared with Milli-Q water. The eluent had a flow rate of 0.5 mL min^{-1} (Hitachi L-2130).

The amount of biomass in both reactors was quantified as cell dry weight (cdw). 5 mL of sample were filtrated using previously dried and weighed 1.0 µm pore size filters with vacuum filtration. Membranes were placed in the oven at 105 °C for 24 hours. After

cooling down, they were weighed, and the biomass concentration was determined in g L^{-1} of TSS. Afterward, the membranes were submitted to 550 °C for 2 h and weighed after cooling to determine VSS in g L^{-1}.

COD was measured with Spectroquant Kit (Merck) and the solutions used were prepared according to *Standard Methods* (Clesceri et al., 1999).

The monitoring of LS content was performed according to Restolho et al. (2009). The measurement was performed using a UV Spectrophotometer at 275 nm, after a dilution of 1:10. The lignosulphonates concentration was calculated resorting to the Beer-Lambert law, considering a molar absorptivity of 7.41 g^{-1} cm^{-1} (Restolho et al., 2009; Xavier et al., 2010).

PHA cell content was determined using GC according to Moita et al. (2012). The pellet collected after the centrifugation of 1.5 mL of sample was lyophilized. The biomass was incubated with 1:1 solutions of chloroform with heptadecane as internal standard and an acidic methanol solution (20% H_2SO_4), at 100 °C for 3.5 h. After cooling, 0.5 mL of water were used for extraction. The chloroform phase was collected and molecular sieves (0.3 mm) were added to ensure water adsorption. 2 µL of the obtained solution were injected into a gas chromatograph coupled to a Flame Ionization Detector (GC-FID, Konik Instruments HRGC-3000C). A ZBWax-Plus column was used with hydrogen as the carrier gas (50 kPa) as well as split injection at 280 °C with a split ratio of 1:6 was used. The oven temperature program was as follows: 60 °C; then 20 °C min^{-1} until 100 °C; then 3 °C min^{-1} until 175 °C; and finally 20 °C min^{-1} until 220 °C. The detector temperature was set at 250 °C. Hydroxybutyrate and hydroxyvalerate concentrations were calculated using standards of a commercial P(3HB-*co*-3HV) (88:12, Aldrich) and corrected using an heptadecane internal standard.

10.2.7. Calculations

PHA content was calculated as a percentage of TSS on a mass basis:

$$\% \text{ PHA} = \text{gHA/gTSS} \times 100 \qquad (10.1)$$

Feast to famine ratio (F/F) was calculated dividing the time needed to the consumption of SCOA by the remaining time of the cycle (Serafim et al. 2004).

SCOA volumetric and specific consumption rates (r_{SCOA}; $-q_{SCOA}$), acetic acid volumetric consumption rate (r_{Acet}), propionic acid volumetric consumption rate (r_{Prop}), butyric acid volumetric consumption rate (r_{But}), PHA specific production rates (q_{PHA}), P(3HB) production rates (q_{PHB}), P(3HV) production rates (q_{PHV}) were determined by adjusting linear functions to the experimental data for each variable concentration over time, and calculating the first derivative at time zero. In the case of specific rates each variable was divided by the biomass concentration at that point.

The oxygen uptake rate (OUR) was determined by adjusting linear functions to the experimental data from the respirometer over time, and calculating the first derivative at time zero. The %DO was previously converted to $mgO_2\ L^{-1}$ considering an oxygen saturation concentration at 28 °C of 8.8 $mgO_2\ L^{-1}$. Endogenous OUR was determined before the addition of the substrate using the same method.

PHA production yield on substrate ($Y_{PHA/S}$) was calculated by dividing the amount of PHA by the total amount of substrate consumed. PHA specific productivity was calculated dividing the amount of produced PHA by biomass and time and the volumetric productivity dividing the amount of produced PHA by volume and time.

10.3. Results and Discussion

10.3.1. Selection Step

HSSL was already tested to acclimatize a PHA-storing MMC. However, instability dominated the operational periods most of the time (Queirós et al., 2014) or quite long start-up periods with low PHA accumulation were observed (Queirós et al., 2016). Later, supplementation of HSSL with SCOA allowed obtaining a stable process after 25 days with significant PHA accumulations (Chapter 6).

In this work, an SBR was operated for 156 days and the culture selection was achieved using ADF conditions and fermented HSSL. The acidogenic fermentation was performed by an aerobic CSTR system previously described (chapter 9). The CSTR was operated without pH control, at 30 °C with and HRT and SRT equal to 2.34 days. The main

products of the effluent were acetic, propionic, butyric and valeric acids, (chapter 9). Later, a second stream was collected from a second CSTR with pH control to manipulate the SCOA profile (chapter 9). This last stream was only used for accumulation batches.

During the operational period of the selection SBR, several parameters were monitored: SCOA uptake; PHA production; biomass concentration; F/F ratio; COD and ammonium uptake. Those were used to evaluate the MMC's adaptation to the imposed conditions and its capacity to accumulate PHA. Their evolution was used to choose the operational conditions that allow increasing the selective pressure in the SBR reactor.

The selection step should be a compromise between PHA accumulation capacity and biomass growth, since the volumetric productivity of the accumulation step is dependent not only on the ability of the MMC to accumulate PHA but also on cell density (Albuquerque et al., 2010; Oehmen et al., 2013; Reis et al., 2011; Villano et al., 2010). Since biomass growth and PHA production are processes that compete for the carbon source (Serafim et al., 2008a), the manipulation of OLR is a key factor for the process. In this case, OLR was kept low in the beginning of the process to allow the MMC adaptation towards this toxic substrate and, at the same time, ensure a high selective pressure for PHA storage. Then, OLR was gradually increased when the MMC seemed to achieve an apparent stationary phase, to promote an increase in biomass concentration, without losing PHA production ability. For this reason, during the operational time, parameters such as cycle length and OLR were changed. Thus, three periods of operation can be defined: P1) 24 h cycles with an OLR of 2.2 gCOD L^{-1} d^{-1} until day 26; P2) 12 h cycles with an OLR of 4.5 gCOD L^{-1} d^{-1}, from day 26 until day 90; and finally, P3) 12 h cycles with an OLR of 7.0 gCOD L^{-1} d^{-1} from day 91 until day 156 (Table 10.1).

F/F ratio, biomass concentration, and PHA production were the parameters that gave the best indication of how effective was the selection process (Fig.10.2).

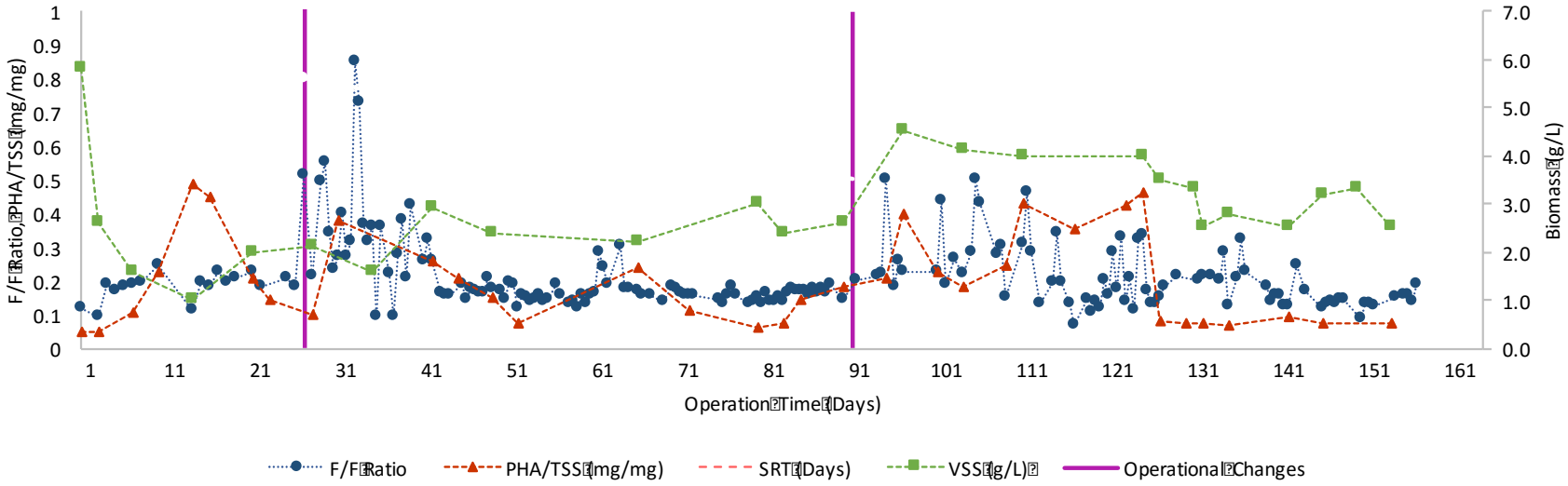


Fig. 10.2. SBR Performance: Evolution of F/F, biomass concentration and PHA content over the operational time.

A correlation between F/F ratio and the PHA accumulation ability of the MMC was already established in the literature. Several studies reported that cycles with feast phase under 20 – 25% of the total cycle duration are associated with good PHA storage response. In contrast, in longer feast phase, PHA storage played a negligible role and the substrate was diverted mainly towards growth (Albuquerque et al., 2010; Reis et al., 2011).

Since the beginning and during the first stage of operation (P1), F/F quickly stabilized around 0.19. The cycle length was then shortened to 12 h, and consequently the HRT decreased to 1 day and OLR increased to 4.5 gCOD L^{-1} d^{-1}. This change resulted in an unstable period, with F/F values ranging from 0.09 to 0.85 (Fig. 10.2). By 42nd day of operation until the 89th F/F values decreased and remained constant around 0.16 (Fig. 10.2). This value represents a good indicator of PHA accumulation capacity of the selected MMC. On the 90th day, OLR was increased to 7.0 gCOD L^{-1} d^{-1}, which led to a new stage of adaptation to the conditions imposed. During this last period, F/F ratio was kept around 0.2. All the adaption periods took no longer than 4.5 SRT, revealing the MMC dynamics and capacity to adapt towards the new conditions imposed.

The F/F values obtained were coherent with those found in literature since several works using ADF as a selection strategy showed PHA accumulation behavior for F/F under 0.2. Dionisi et al. (2006) tested the effect of the OLR on the performance of an SBR fed with a mixture of SCOA and reported F/F ratios under 0.25 for good accumulation capacity, 29.2 ± 4.0% cdw (Dionisi et al., 2006). Johnson et al. (2009) obtained very low F/F ratios (0.1) when the selective pressure imposed on the culture selection SBR fed with acetic acid as sole carbon source was maximized and resulted in PHA accumulation of 53% cdw in SBR cycle and 89% cdw during fed-batch accumulation. Morgan-Sagastume et al. (2015), using a complex substrate, municipal wastewaters, obtained F/F ratios of 0.21. Ben et al. (2016) reported F/F ratios of around 0.15 for an SBR fed with fed fermented brewery wastewater (Ben et al., 2016).

About biomass concentration, there was an increase in the selection reactor throughout the operational time, showing that gradual increases of OLR, despite the toxicity of HSSL, was to a certain extent a successful strategy. Similar values to those

observed were reported when working with brewery wastewater to select a PHA accumulating culture (Ben et al., 2016) or using fermented sugar molasses (Albuquerque et al., 2010). Oehmen et al. (2013) used pH control as a strategy to increase the biomass growth rate while maintaining the specificity of the enrichment for PHA-producing organisms. These authors reported biomass concentrations up to 8 gVSS L^{-1} with pH control at 8, one of the highest biomass concentration in a selection reactor (Oehmen et al., 2013).

PHA accumulation followed the same tendency observed for F/F ratio, P3. After every operational parameter changed, erratic accumulations were observed but after a certain period, the values stabilized. For P2, after F/F stabilization, PHA varied 12.7 ± 6.5% cdw. From day 126 onwards (P3), the PHA content remained constant at 7.8 ± 0.7% cdw. Regardless of the imposed conditions, and the changes on PHA content, this parameter tended to the same values after culture adaptation. This was consistent with the other data collected and indicated that the culture reached a pseudo-stationary state (PSS). Analogous behavior was reported Valentino et al., (2014) and Villano et al., (2014). Several authors also reported similar polymer contents values to those obtained in this study. Villano et al. (2014) reported an average concentration of 15% for the period of stable operation of an SBR reactor fed with acetic and propionic acids mixture was reached. Moita and Lemos (2012), using bio-oil as substrate, found a PHA content in the selection SBR of 9.2%. Low contents were also observed by Morgan-Sagastume et al. (2015) when operating a pilot-scale reactor integrated into a municipal wastewater treatment plant that selected an MMC with biomass PHA contents below 4%. Also with HSSL, similar value was obtained by Queirós et al. (2016) with HSSL. On the other hand, several papers also described higher values, Albuquerque et al. (2010) showed an average PHA content of 25% with sugar molasses as carbon source, and Chen et al. (2016) operated four SBRs with different SRT fed with fermented sugar cane wastewater and reported PHA contents between 9.55 and 30.75%.

A quite important factor, especially for downstream process, is the composition of PHA produced and their stability. It is highly desirable to know how to manipulate it and find the corresponding applications (Laycock et al., 2013). For this reason, the monomeric

composition of PHA accumulated during the SBR operation was also evaluated and is presented in Fig. 10.3 and Table 10.3.

The results showed that during all the operational time a copolymer of P(3HB-*co*-3HV) was produced, which was expected since the carbon source had precursors for both monomers (Lemos et al., 2006). During P1, 3HB was the predominant monomer and its percentage in the polymer remained relatively constant especially after stabilization (6th day), with an average of 65 ± 6.3% of 3HB. The change in the cycle duration had no visible impact on PHA composition. Nonetheless, after stabilization on the 44th day, there was a small decrease in the 3HB content to an average of 62%. After the change of the OLR on the 90th day, an increase of the 3HB content was observed, from 62% to 78%, and followed by a sudden prevalence of 3HV, 28%. After that, 3HB content rapidly increased to previous values, having achieved its highest percentage on the 153rd day, 83%.

Molar fractions of 3HB and 3HV remained rather stable during the various PSS, which is advantageous since more stable copolymers production is desirable. The cause for typical variations on PHA composition is usually attributed not to the fact that an MMC or a complex substrate is being used, but to the SCOA profiles variations fed to SBR (Lemos et al., 2006; Wang et al., 2013). The acidogenic fermentation took place without pH control and because of that the effluent collected presented variable SCOA profiles (chapter 9). Consequently, every time the feed was renewed, a different SOCA profile was fed to the selection reactor. Due to the constant dominance of acetic acid as the main fermentations product (plus the butyric acid) 3HB was the predominant monomer. Previous works with HSSL reported accumulation of glucose biopolymer due to the high sugar fraction of the liquor (Queirós et al., 2016), allowing a side population to develop and decrease the PHA accumulation and yields of the process. Although some xylose was not converted into SCOA and, consequently, being present in the effluent from the acidogenic reactor, no GB was observed.

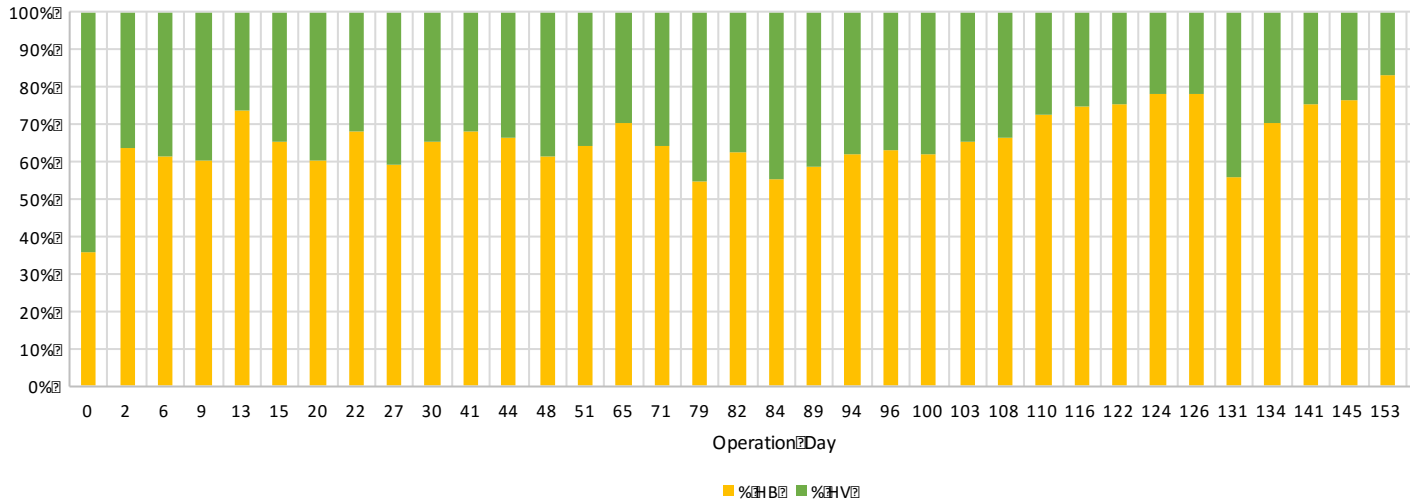


Fig. 10.3. Monomer composition through the operational.

Table10.3. Kinetic and stoichiometric parameters obtained in the SBR cycles.

Period	Day	F/F	X	r_{SCOA}	r_{Acet}	r_{Prop}	r_{But}	q_{VolPHA}	q_{pPHA}	q_{HB}	qp_{HV}	HB:HV	$Y_{PHA/S}$	$Prod_{Esp}$	$Prod_{Vol}$
P1	13	0.26	1.1	6.27	3.41	1.52	2.10	0.169	0.162	0.152	0.019	64:36	76%	0.244	0.244
P2	48	0.08	2.0	10.08	9.55	2.66	3.98	0.070	0.030	0.045	0.027	65:35	46%	0.148	0.354
P3	141	0.13	3.2	10.45	6.48	3.97	-	0.030	0.014	0.026	0.010	63:37	55%	0.061	0.152

X – gL^{-1}; r_{SCOA} – Cmmol L^{-1} h^{-1}; q_{Acet}– Cmmol L^{-1} h^{-1}; q_{But}– Cmmol L^{-1} h^{-1}; q_{VolPHA} – CmmolPHA L^{-1} h^{-1}; $\mathbf{q_{EspPHA}}$ – CmmolPHA $CmmolX^{-1}$ h^{-1}; q_{HB}– CmmolHB L^{-1} h^{-1}; q_{HV} – CmmolHV L^{-1} h^{-1}; HB:HV – molar fraction; $Y_{PHA/S}$ – CmmolPHA $CmmolS^{-1}$; $Prod_{Esp}$ – gPHA gX^{-1} h^{-1}; $Prod_{Vol}$– gPHA L^{-1} h^{-1}

P(3HB-*co*-3HV) production in selection SBRs using real feedstocks is widely documented (Albuquerque et al., 2010; Ben et al., 2016; Jiang et al., 2012, 2009; Villano et al., 2014). Similar monomer proportions to those of this study were reported by Moita and Lemos (2012) using bio-oil and Oehmen et al. (2014) with fermented molasses without pH control. Queirós et al. (2016) obtained a copolymer of P(3HB-*co*-3HV) with 20% of 3HV using non-fermented HSSL. The higher 3HV fraction obtained could be the result of the introduction of a pre-fermentation step. This resulted in a wider variety of SCOA or a higher production of organic acids precursors for 3HV, as propionic and valeric acids.

SBR cycles were monitored periodically to evaluate the MMC performance. Several parameters were followed, namely the concentrations of SCOA, PHA, biomass, COD, ammonium and LS as well as DO percentage and pH. Kinetic and stoichiometric parameters of three representative cycles of each period (P1 – P3) are represented in Table 10.1. Fig. 10.4 depicts a representative cycle of P3 PSS. In general, the same behavior was observed for all periods once PSS was reached.

In all cycles, even if at different rates, there was a clear substrate preference for acetic acid over the other acids (Table 10.3). This carbon source preference could be explained by the metabolic pathways for the different acids (Lemos et al., 2006). Propionic acid can lead to the production both 3HB and 3HV. To produce 3HV, propionic acid is converted to propionyl-CoA and condensate with acetyl-CoA. To obtain the necessary acetyl-CoA from propionic acid, propionyl-CoA must enter the TCA via methylmalonyl-CoA interconvertion to succinyl-CoA. Unlike acetic acid that directly originates acetyl-CoA, propionic acid consumption is dependent on the rate of decarboxylation of propopionyl-CoA, which can explain its lower rate. Butyric acid also requires extra steps to be converted into P(3HB) or acetyl-CoA, when compared to acetic acid, since acetyl-CoA is a necessary precursor for cell metabolism acetoacetyl-CoA cleavage to acetyl-CoA may be required (Fradinho et al., 2014).

SCOA were consumed at different volumetric rates, acetic acid at 6.48 CmmolAcet L^{-1} h^{-1} and propionic acid at 3.97 CmmolProp L^{-1} h^{-1}. Acetic acid was the preferred acid during all selection SBR operation (Table 10.2). Nevertheless, an apparent preference

towards propionic and butyric acids was taking place during the last selection conditions imposed to the MMC (Table 10.3).

The depletion of the acids was marked by a sharp increase in the %DO (Fig. 10.4) and by the maximum of PHA accumulation with a storage content of 9% cdw and a production rate of 0.014 CmmolHA L^{-1} h^{-1}.

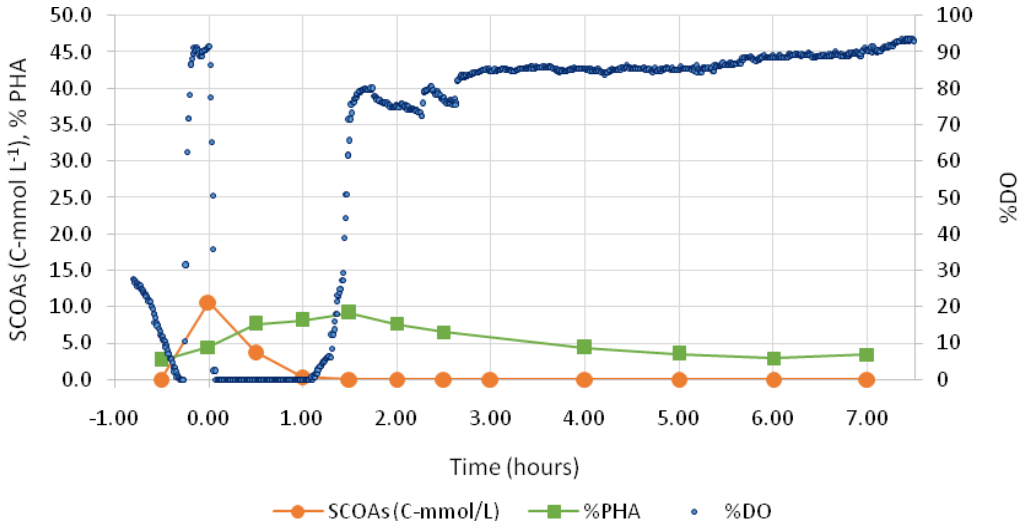


Fig. 10.4. Representative cycle of P3 PSS. Evolution of %DO, SCOA and %PHA.

As expected, due to the conditions imposed, the MMC started with high storage yields and PHA production rates (Table 10.3). Combination of long cycle lengths and low OLR led the MMC to direct the carbon to PHA accumulation instead of growth. Due to the long starvation period, the culture needed more PHA to survive than in shorter cycles. The imposition of several cycles with these conditions steered the culture towards high PHA accumulation rates and content (Table 10.3). As the OLR was increased, either through cycle manipulation (P2) or feed concentration (P3), the MMC started to develop cellular machinery to divert more carbon source to growth while PHA was accumulated at slower rates (Table 10.3). Nonetheless, a good accumulation response was maintained, allowing to increase the volumetric productivity in the next step once more biomass was being formed. Even with the decrease of PHA production during the operation, the values obtained for the specific production rate in the presented cycles were higher to those

reported by Queirós et al. (2016), 0.03 – 0.04 CmmolHA $CmmolX^{-1}$ h^{-1}, using the same substrate and in the range of those observed in the literature 0.05 – 0.28 (Albuquerque et al., 2010; Moita and Lemos, 2012; Oehmen et al., 2013).

Apparently, SCOA were the only carbon source consumed. Although there were other compounds liable to be consumed, like lignosulphonates and phenolics, their complexity and the reaction time made them unlikely to be used. LS consumption was not verified in any cycle monitored, even though a slight use, possible from the microbial community with no accumulation capacity, was reported in previous works with the same substrate (Queirós et al., 2014). Nonetheless, LS concentration increase, from 3 g L^{-1} to 5 gL^{-1} consequence of OLR increase, and seemed to have no inhibitory effect on the MMC.

10.3.2. Accumulation Step

After successfully obtaining an enriched MMC in PHA-accumulating organisms in the selection process, the accumulation step could be performed to maximize PHA production and achieve the highest PHA content volumetric production rate as possible.

Nine accumulation assays, under fed-batch conditions, were performed to better understand the influence of the selection conditions over the accumulations step. Tests were made under different conditions, as presented in Table 10.3.

The MMC selected in the previous step was collected from the SBR and inoculated to a batch reactor and fed in consecutive pulses to potentiate PHA accumulation and avoid inhibition by substrate. A new pulse was added after a sudden increase in the %DO in the reactor, indicating substrate depletion.

One of the goals of this work was to integrate different by-products from CAIMA in a three stage PHA production process, namely HSSL and Condensate. Since the main carbon source in Condensate is acetic acid this substrate was only used in the last stage of the process. It was expected that an MMC selected using a mixture of SCOA could use only acetic acid to accumulate PHA. Nonetheless, Condensate has in its composition other potential inhibitors, such as methanol and furfural, to which the MMC was not adapted and that could undermine the accumulation process.

Two distinct sets of assays can be defined, the first with biomass from the PSS of

P2 and the second from the PSS of P3. For all tests, several kinetic and stoichiometric parameters were calculated and are presented in Table 10.3.

10.3.2.1. Accumulations Batches from P2

AT1 – AT4, in general, presented similar performance, accumulating around 45% cdw PHA. In all tests, OUR became closer to the endogenous value over time, and the acids volumetric uptake rate decreased after each pulse was given. Since endogenous OUR corresponds to the oxygen needed for cell maintenance, the lowering of the oxygen demands could be an indication that the MMC was not metabolizing the carbon source for PHA accumulation. This could mean that the MMC achieved the maximum storage capacity. This is further demonstrated by the decrease of consumption rate of SCOA in each pulse, calculated posteriorly and by the determination of the PHA content.

The assays with ammonium limitation presented the highest accumulation and $Y_{PHA/S}$, 51.2% and 0.14 CmmolPHA CmmolSCOA^{-1}, respectively, for Stream A, and 46.5% and 0.25 CmmolPHA CmmolSCOA^{-1} for condensate (Table 10.4). However, the storage contents obtained were not significantly higher when compare to the assays without limitation (45.2% and 43.2% for Stream A and Condensate, respectively), being the product formation rate even higher in these cases. Possibly, the duration of the accumulation without limitation was not enough for the MMC to replenish the cellular machinery necessary for growth and so the PHA content achieved was comparable.

Polymer composition manipulation was shown to be possible when different streams were fed to the culture due to their SCOA content. With acidified HSSL, a P(3HB-*co*-3HV) was obtained, whereas condensate gave origin to P(3HB).

A strong impact of ammonium limitation was observed regarding volumetric productivity. The MMC under no limiting conditions consumed the same amount of carbon 3 times faster and produced PHA 2.5 times faster than the MMC under ammonium limitation. This factor combined rebound on volumetric productivities, doubling it.

To understand the role of acetic and propionic acids, the main SCOA produced in the acidification process, on PHA storage, accumulation assays using synthetic acids and

the HSSL matrix were performed. Each acid was tested separately by the addition of 35 Cmmol of acid per pulse to a batch reactor. The assay with acetic acid showed the clear preference towards this acid, with volumetric consumption rates between 20 – 30 Cmmol $L^{-}1\ h^{-1}$. The behavior was very similar to assays with Stream A and Condensate. Besides, with acetic acid being the only carbon source present, a quicker accumulation was expected since the consumption rate for this SCOA was always the highest in both the selection SBR and the previous accumulation assays. For these reasons, the performance of this assay was better than those before with a maximum accumulation of P(3HB) of 43.6%, a yield of 0.14 $CmmolPHA\ CmmolS^{-1}$, and the highest volumetric production of all assays (Table 10.4). The results also indicate that the MMC could probably still accumulate more polymer since the OUR did not reach the endogenous value and the consumption rates did not enter the downturn in consumption (data not shown).

Regarding the assay with propionic acid, the data collected showed an interesting behavior as the MMC seemed to adapt to the carbon source over time, consuming it faster at each pulse. In fact, OUR also increased over time, which was the opposite tendency to what was observed in previous assays, and a sign of higher substrate metabolization. Some works have already focused on the behavior of MMC submitted to a substrate shift and their substrate preferences (Carvalho et al., 2014; Lemos et al., 2006). Lemos et al. (2006) selected two different MMC with acetic and propionic acids and then fed them with other SCOA and a mixture of both. They observed that the MMC was capable of accumulating PHA, even if in some cases the SCOA uptake rates were inferior. They also found that cultures selected with different substrate sources produced various copolymers when feeding the same SCOA mixture.

Table 10.4. Conditions and kinetic parameters of the accumulation tests performed.

Assay	Period	Culture	Substrate	Limitation	q_{SCOA}	q_{PHA}	%PHA	HB:HV	$Y_{PHA/S}$	ProdEsp	ProdVol	Reference
AT1	P2	MMC	Stream A	None	1.67	0.18	45.2	72:28	0.10	0.17	0.20	This work
AT2	P2	MMC	Stream A	Ammonium	0.48	0.07	51.2	76:24	0.14	0.07	0.10	
AT3	P2	MMC	Condensate	None	0.93	0.23	43.2	100:0	0.24	0.10	0.21	
AT4	P2	MMC	Condensate	Ammonium	0.42	0.11	46.5	100:0	0.25	0.10	0.06	
AT5	P2	MMC	Matrix + Ac	None	0.90	0.13	43.6	100:0	0.14	0.15	0.28	
AT6	P2	MMC	Matrix + Prop	None	0.24	0.03	26.5	61:39	0.14	0.03	0.08	
AT7	P3	MMC	Stream A	Ammonium	0.35	0.07	74.4	74:26	0.20	0.07	0.27	
AT8	P3	MMC	Stream B	Ammonium	0.26	0.03	40.7	67:33	0.13	0.03	0.12	
AT9	P3	MMC	Condensate	Ammonium	0.46	0.01	9.6	100:0	0.02	0.01	0.02	
		MMC	HSSL	None	---	---	63.3	100:0	0.77*	0.16	0.14	Queirós et al. (2014)
		MMC	HSSL	None	---	0.09	4.6	80:20	0.12	0.01	0.02	Queirós et al. (2016)
		MMC	HSSL	N	---	---	34.6	76:24	0.79	0.07	---	Chapter 6
		MMC	Fermented paper mill wastewater	N	---	---	76.8	86:14	0.76*	0.15	0.08	Jiang et al. (2012)
		MMC	Fermented sugar molasses	N	---	---	74.6	74:26; 83:17	0.81	0.49	1.50	Albuquerque et al. (2010)
		MMC	Fermented molasses	None	---	---	57.5	90:10	0.57-0.65	---	10.8	Oehmen et al. (2014)
		MMC	Syntetic acetic acid	N	---	---	89.0	100:0	0.6	1.2	---	Johnson et al. (2009)
		C. necator	Demethanolized crude glycerol phase	None	---	---	64.5	100:0	---	---	0.98	Špoljarić el al. (2013)
		R. *E. Coli*	Whey (ultrafiltration, enzymatic hydrolysis)	None	---	---	87.0	100:0	---	---	4.6	Ahn et al. (2001)
		H. mediterranei	Extruded rice bran	None	---	---	55.6	87:13	---	---	0.65	Huang et al. (2006)

Units: X – g L^{-1}; q_{SCOA}–CmmolSCOA $CmmolX^{-1}$ h^{-1}; q_P – CmmolPHA $CmmolX^{-1}$ h^{-1}; $Y_{PHA/S}$ – CmmolPHA $CmmolS^{-1}$; $Prod_{Esp}$ – gPHA gX^{-1} h^{-1}; $Prod_{Vol}$ – gPHA L^{-1} h^{-1}; *gPHA $gCOD^{-1}$

Even though substrate intake improved during the test, this was not translated into an increase in accumulation over time, reaching only 25% of PHA content. The low accumulation could be the result of a small fraction of microorganisms that could use propionic acid. As the MMC was selected using a mixture of SCOA dominated by acetic acid, shifting the feed to propionic acid alone will originate a strong competition inside microbial community for substrate utilization than for mixed-carbon-acclimated sludge. Due to this competition, substrate utilization efficiency is reduced and together the amount of PHA accumulated (Wang et al., 2013).

Regarding PHA monomeric composition, as expected, the use of propionic acid resulted in the production of two monomers with the highest 3HV fraction of all accumulation assays, 61:39 (HB:HV).

10.3.2.2. Accumulations Batches from P3

Since these assays used biomass from P3, the initial concentration of biomass was higher and the amount of SCOA in each pulse was 50 Cmmol to mimic the conditions of the selection SBR. The tests were conducted under ammonium limitation since better PHA contents were achieved under this conditions in the tests using an MMC from P2. Moreover, literature as showed that this limitation promotes accumulation in processes using real substrates (Albuquerque et al., 2010; Villano et al., 2014) and HSSL (chapter 6).

Eight pulses with approximately 50 Cmmol of SCOA of Stream A were given at AT7 (Fig. 10.5), with a performance similar to AT2. Nonetheless, some interesting aspects can be highlighted. First, two distinct increases in the variation of the %DO could be observed in each pulse. A lower one almost right after the addition of the pulse and another later and more evident corresponding to the SCOA exhaustion (Fig. 10.5). Since the method used was not able to detect all the SCOA due to low concentration, it was possible that the first increase was related to the depletion of one of the non-detected SCOA.

The type of copolymer produced was comparable to AT1, being produced a copolymer of P(3HB-*co*-3HV) with a molar fraction of HB:HV of 74:26, showing reproducibility among the accumulation tests. In addition, the strategy adopted for the MMC selection led to an increase in all the main stoichiometric parameters with an $Y_{PHA/S}$

of 0.20 CmmolPHA $CmmolS^{-1}$, volumetric productivity of 0.27 gPHA L^{-1} h^{-1} and a maximum content of 74.4% comparable to the highest values reported in the literature with MMC and waste/surplus feedstock (Fig. 10.5, Table 10.4) (Albuquerque et al., 2010; Jiang et al., 2012). Regarding the PHA content, a closer look at OUR evolution should be given. The assay was prolonged after the OUR stabilization near OUR endogenous and a decrease of the PHA content was observed (Fig. 10.5).

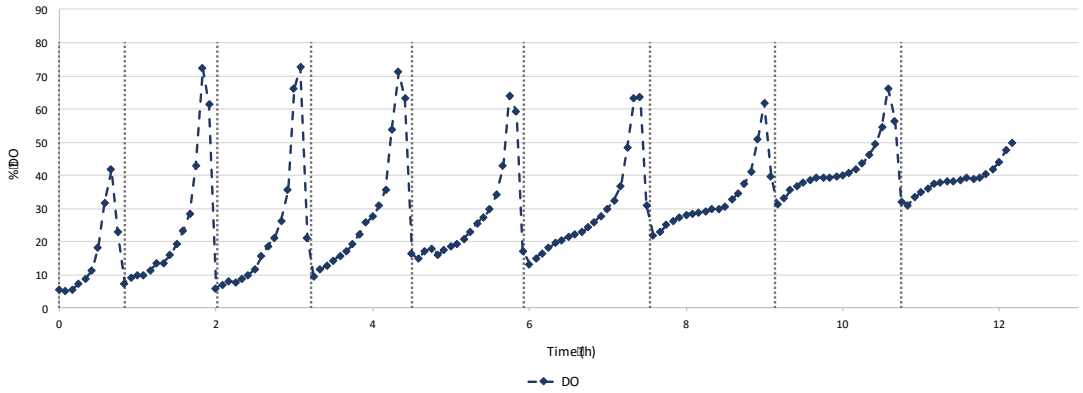
Using Stream B, AT8, the MMC had a very similar response to AT7. As said before, the main difference between these two streams is the relative SCOA composition. Given this, and although the MMC was not acclimatized to this substrate, inhibitory effects were not expected. Nonetheless, some interesting aspects can be highlighted, in the variation of the %DO, again two distinct increases could be observed in each pulse. The existence of an unknown compound in stream B, suspected to be succinic acid, seemed not to interfere in the PHA accumulation, being even accumulated during the test (chapter 9).

One of the main difference regarding the use of stream B was the monomeric composition of the PHA produced, with 67:33 of HB:HV, showing the possibility of manipulating the PHA composition through the manipulation of acidogenic operation as it was already reported by Albuquerque et al. (2007). However, when compared to the accumulation using stream A, lower accumulation and volumetric productivities were observed, 40.7% cdw and 0.12 gPHA L^{-1} h^{-1}, respectively.

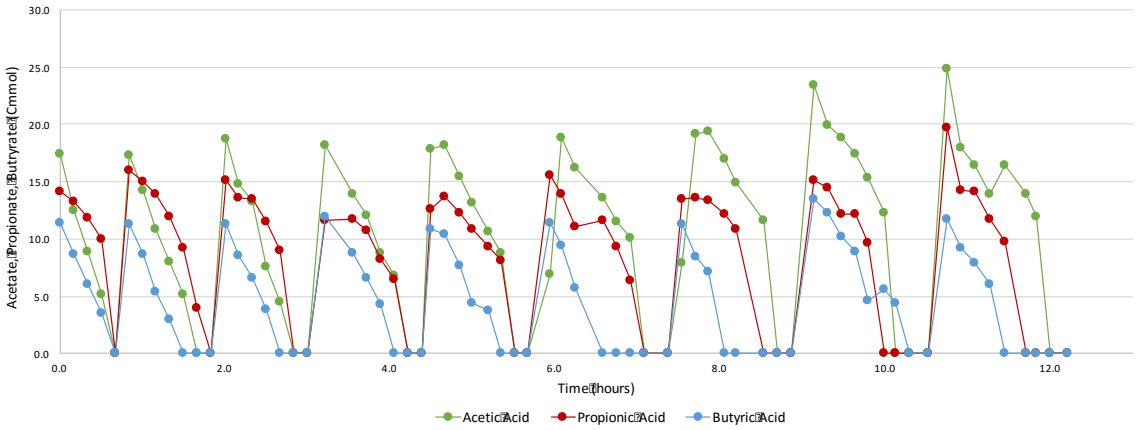
The assay AT9 was conducted with Condensate in pulses of 50 Cmmol of acetic acid. In the previous test with this substrate, AT3 and AT4, the MMC presented significant accumulations and production ratios and the tests showed room for improvement since the MMC apparently had not reached polymer saturation (Table 10.4). Based on this, more concentrated pulses were added in the assay. Even though the test followed the same tendencies as the previous ones, it underperformed. The consumption rates calculated for this test were similar to those achieved in the other assays with Condensate. However, the substrate was not being directed towards P(3HB) production, since the values of polymer content in the cells were the lowest of all assays. By the end of this test the culture had accumulated less than 20% of P(3HB) with a yield of 0.02

CmmolPHA $CmmolS^{-1}$.

a)



b)



c)

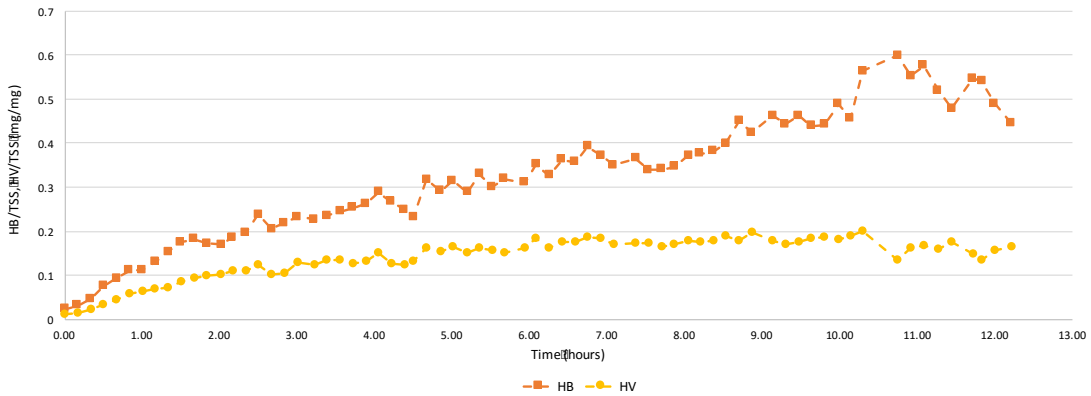


Fig. 10.5. Accumulation Test with Stream A, during P3. a) %DO profile; b) SCOA consumption; c) 3HB and 3HV accumulation.

This result was not expected since the use of Condensate as substrate for PHA production using the selected MMC was already validated in assays AT3 and AT4 and

although the biomass had a different origin (in this case it was from Period 3 of the SBR operation), the same culture had already shown good accumulation capacity in tests AT7 and AT8. The most likely explanation for the obtained results is the inhibitory effect of the compounds present in the Condensate. To enable the addition 50 Cmmol of acetic acid, the pulses were concentrated in these assays. This increase resulted in a higher concentration of inhibitory compounds present in the Condensate, methanol and furfural around 0.33 g L^{-1}. Literature shows that if not acclimatize to high concentrations of these compounds, PHA accumulation would be severe inhibited (Moita et al., 2014).

Finally, the MMC could accumulate PHA with all substrates fed, even those with different composition than the one it was selected in. This shows the importance of the selection with a mixture of SCOA, since mixed-carbon-acclimated cultures are quicker to adjust to substrate variations as observed by Wang et al., (2013). Microorganisms have different metabolic pathways that allow for the use of different carbon sources, the enrichment of the culture in an SCOA mixture leads to a diverse microbial composition and ensures better substrate utilization efficiency for every SCOA fed (Carvalho et al., 2014; Lemos et al., 2006; Wang et al., 2013). Higher SCOA consumption rates and PHA cell were evident in the assays conducted under nitrogen limitation. Such restriction was an important parameter in the accumulation process since it limits cell growth and carbon flow is diverted towards PHA production.

Johnson et al. (2009) using acetic acid as feed in the accumulation process reported the best polymer content achieved with MMC, of 89% cdw. However, considering the best results obtained in this work, they are comparable to those obtained in other works using MMC and complex substrates. Jiang et al. (2012) used fermented paper mill wastewater as carbon source and produced a copolymer of P(3HB-*co*-3HV), with a maximum accumulation of 76.8% cdw and a volumetric productivity of 0.08 gPHA L^{-1} h^{-1}. Using fermented sugar molasses, Albuquerque et al. (2010) reported a maximum accumulation of 74.6% cdw and a volumetric productivity of 1.50 gPHA L^{-1} h^{-1}. Oehmen et al. (2014) reported one of the highest volumetric productivities with MMC and real substrates, 10.8 gPHA L^{-1} h^{-1} with fermented molasses due to a high biomass concentration, 11.8 g L^{-1}. These results show that although the PHA content achieved in

this work was comparable to the literature, there is still room for improvement regarding the volumetric productivity. As Table 10.4 shows, this work has some of the lowest yields of $Y_{PHA/S}$ compared to other works in the area and to the values obtained in the selection step. This may be explained by the complexity and toxicity of HSSL. In these assays, the OLR fed to the reactor was much higher than in the selection step, and while SCOA were being consumed, other compounds were being accumulated with each pulse. Because of the presence of phenolic compounds of low molecular weight in the medium that could diffuse to the interior of the cell, some of the carbon could be diverted to energy production to maintain intracellular stability (Pereira et al., 2012).

10.4. Conclusion

This work explored different pulp and paper factory residue streams for PHA-storing MMC selection and to manipulate PHA composition. A three-step process was adopted and HSSL was the substrate chosen. In the first step HSSL was submitted to acidogenic fermentation in different conditions to originate two distinct streams of effluent with distinct SCOA composition that were used in the subsequent steps.

Good results were achieved using each substrate source proposed at the beginning of this work. The selected MMC accumulated a maximum of 74.4% cdw of PHA copolymer using Stream A with a volumetric productivity of 0.27 gPHA L^{-1} h^{-1} and a $Y_{PHA/S}$ yield of 0.20 CmmolPHA $CmmolS^{-1}$ (AT7). Stream B had a different SCOA profile which leads to the production of the copolymer with a different 3HV content. A maximum PHA of 40.7% cdw was obtained in AT8 with a volumetric production of 0.12 gPHA L^{-1} h^{-1} and a $Y_{PHA/S}$ yield of 0.13 CmmolPHA $CmmolS^{-1}$. Finally, Condensate led to the production of homopolymer of P(3HB) with a maximum of 43.2% and a volumetric production of 0.21 gPHA L^{-1} h^{-1} and a $Y_{PHA/S}$ yield of 0.24 CmmolPHA $CmmolS^{-1}$.

The MMC capacity to adjust to the operational conditions and variations of the process ensures the success of its use in dynamic systems fed with complex substrates. HSSL and Condensate are currently sold to produce new chemicals or burned for energy production. And so, their inclusion in a biorefinery concept with PHA production has the

potential to be a more economically and ecologically sustainable solution for the pulp and paper industry.

10.5. References

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Chapter 11

General Conclusions and Future Prospects

11.1. General Conclusions

A three-step process for PHA production by MMC using HSSL as carbon source was successfully established.

The acidogenic fermentation was studied in some detail. Two distinct reactor configurations, a CSTR with and without pH control, and an MBBR without pH control were set up to carry out the acidogenic fermentation. Regarding the CSTR configuration, type of inoculum, pH control, and HRT were studied to understand how they could influence the concentration and profile of SCOA produced.

By starting with an aerobic culture, it was possible to obtain an MMC enriched in facultative anaerobic acidogenic microorganisms. When operating without pH control, the culture took 46 days to adapt to the conditions imposed, reaching an AD_{Total} of 38%. The main acids produced in all conditions were acetic, propionic, butyric, valeric and lactic acids. By controlling the pH, shorter adaptation periods were required and a more stable profile of SCOA was observed, lowering, however, the acids concentration. Nevertheless, it was possible to control the profiles obtained. Lastly, an MBBR without pH control was studied with the objective of retaining the biomass in the system. Changing the reactor configuration was possible to reach quicker a PSS with sugar being entirely consumed and with a stable profile produced. Also, the dominant acid shifted from acetic to butyric acid.

Fermented HSSL revealed to be a suitable feedstock to enrich an MMC in PHA-storing microorganisms and maximize the production. An initial study with two-step process using HSSL, without being fermented, to select an MMC showed to be unstable along the operational period or required a quite long start-up periods. A low PHA accumulation was always observed undermining the possibility of establishing a PHA production process directly from HSSL. Moreover, an extensive microbiological characterization showed a very dynamic bacterial community, even during more stable periods and with an active side population unable to accumulate PHA. Then, the following study was conducted with HSSL supplemented with SCOA. A stable process was established after 25 days with significant PHA accumulations, and through cycle length and OLR manipulation, it was possible to tune the selection conditions and reach an MMC dominated by known PHA-producers, namely the genus *Acidovorax*.

Due to the set of results obtained, eco-engineering of MMC with fermented HSSL was applied. The MMC could adapt to several imposed conditions, revealing its robustness every time the operational parameters were changed. The F/F ratio was kept below or equal to 0.2, with a constant production of a copolymer of P(HB-*co*-HV). A parallel goal was also accomplished. During the accumulation tests, different streams from the partner pulp and paper industry were used to manipulate the PHA composition. Two different copolymers of P(HB-*co*-HV) were produced from HSSL fermented at different pH. Using condensate rich in acetic acid but containing methanol and furfural, a homopolymer of PHB was obtained. From the stream used during the selection stage, the MMC was able to accumulate up to 74.4% cdw, with a volumetric productivity of 0.27 gPHA L^{-1} h^{-1}. The PHA content attained is among the highest reported in the literature.

This project proved that HSSL could be, in fact, valorized within a lignocellulosic biorefinary concept. Although the main goal of this project was PHA production, SCOA production should also be a technology to look into. Both products present market value and are highly demanded nowadays. In this way, it was proved the potential to insert HSSL in the circular economy concept, broadening its valorization through sustainable microbiological processes.

11.2. Future Prospects

Although the studies conducted along the present PhD project can be considered closed and completed, the research concerning the valorization of HSSL still requires the clarification of several issues:

- Improvement of the biotreatability process of HSSL: new and affordable materials should be tested and the experimental conditions of the application of those materials should be optimized. This could be done, for example, through the functionalization of bentonite and chitosan to make them selective towards LS and phenolic components. Also, applications to such materials after the pre-treatment should be researched;
- Broadening pH control of acidogenic fermentation. Several metabolites, besides the most common SCOA, were produced and not accounted for. Succinic and

fumaric acids could be among them and are two of the most high-valued building-blocks;

- Deepening the knowledge of the influence of different operating conditions on the SCOA produced, namely the combination of high OLR and pH control on the MBBR;
- Evaluation of the influence of microbial populations present in the reactors in the SCOA production and the profiles achieved;
- Regarding the optimization of the MMC selection, different parameters may still be tested, such as SRT, aeration, C/N ratios, different reactor conformation like membrane reactors. Their impact on the selective pressure for PHA storage and/or impact on cell's growth capacity should be studied by monitoring reactor performance and predicting the mechanisms governing culture enrichment using metabolic models;
- Concerning the last adopted strategy, to continue the increase of OLR without causing the F/F ratio to increase should be investigated and understand the factors that hinder significant biomass growth;
- About the accumulation stage: new strategies to improve volumetric productivity need to be considered;
- And finally, an exhaustive study of the properties and characteristics of the polymers produced needs to be addressed. In particular, their thermal properties, through thermogravimetric analysis, and mechanical properties, to determine the tensile strength, Young's modulus of elasticity and viscoelastic behavior but also molecular weight and crystallinity. Still in this consideration, understanding how the HSSL matrix would influence the polymer produced once extracted from the cells would be an important study to be conducted.
- Finally, the study of the microstructures of the PHA polymer chains would also be a relevant work. To understand of how homopolymers, random copolymers, block copolymers, or block-random copolymers can be obtained and impact the final applications of PHA.

Annex A

Fish probes used

Probe	Sequence (5' – 3')	Target	References
EUB338	GCTGCCTCCCGTAGGAGT		
EUB338 II	GCAGCCACCCGTAGGTGT	Bacteria	Amann et al 1995
EUB338 III	GCTGCCACCCGTAGGTGT		
Delta495a	AGTTAGCCGGTGCTTCTT		
Delta495b	AGTTAGCCGGCGCTTCCT	*Deltaproteobacteria*	Loy et al 2002; Lücker et al 2007
Delta495c	AATTAGCCGGTGCTTCCT		
Lgc354a	TGGAAGATTCCCTACTGC	*Firmicutes* (Gram$^+$	
Lgc354b	CGGAAGATTCCCTACTGC	bacteria with low GC	Meier et al 1999
Lgc354c	CCGAAGATTCCCTACTGC	content)	
Gnsb941	AAACCACACGCTCCGCT	*Chloroflexi* (green nonsulfur bacteria)	Gich et al 2002
Alf968	GGTAAGGTTCTGCGCGTT	*Alphaproteobacteria* (except Rickettsiales)	Neef 1997
Bet42a	GCCTTCCCACTTCGTTT	*Betaproteobacteria*	Manz et al 1992
Gam2a	GCCTTCCCACATCGTTT	*Gammaproteobacteria*	Manz et al 1992
Hgc69a	TATAGTTACCACCGCCGT	*Actinobacteria* (high GC Gram$^+$ bacteria)	Roller et al 1994
Pla46	GACTTGCATGCCTAATCC	*Planctomycetales*	Neef et al 1998
Cf319a	TGGTCCGTGTCTCAGTAC	*Flavobacteria, Bacteroidetes, Sphingobacteria*	Manz et al 1996
Arc915	GTGCTCCCCCGCCAATTCCT	*Archaea*	Stahl 1991
TM7905	CCGTCAATTCCTTTATGTTTTA	Candidate division TM7	Hugenholtz et al 2001
DF988*	GATACGACGCCCATGTCAAGGG	*Defluvicoccus*	Meyer et al 2006
DF1020*	CCGGCCGAACCGACTCCC		
TFO-DF218	GAAGCCTTTGCCCCTCAG	*Defluvicoccus* related TFO	Wong et al 2004
TFO-DF618	GCCTCACTTGTCTAACCG		
SBR9-1a	AAGCGCAAGTTCCCAGGTTG	*Sphingomonas*	Beer et al 2004
THAU646	TCTGCCGTACTCTAGCCTT	*Thauera sp.*	Lajoie et al 2000
AZO644	GCCGTACTCTAGCCGTGC	*Azoarcus sp.*	Hess et al 1997
PAR651	ACCTCTCTCGAACTCCAG	*Paracoccus*	Neef et al 1996
AMAR839	CCGAACGGCAAGCCACAGCGTC	*Amaricoccus sp.*	Maszenan et al 2000

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